The structure and development of mammalian enamel.

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THE STRUCTURE AND DEVELOPMENT OF MAMMALIAN ENAMEL.

Thesis submitted by

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ABSTRACT

Enamel development and structure have been studied in a number of placental and maraupial mammals, by light microscopy; electron-microscopy; and scanning electron microscopy. The relationship between the formative cells of the enamel and its structural organisation into "prisms" and interprismatic regions has been studied in particular. The crystallites in developing enamel tend to be oriented perpendicular to its mineralising front; but their orientation may be modified by either the translatory movement which may occur between certain surfaces of the TOMES' processes of the ameloblasts and the mineralising front, or the self directed growth of groups of groups of crystallites. The presence of a repetitive (prism) pattern of crystallite orientation in formed enamel is determined by changes of orientation of and within the mineralising front: these changes are 1) the result of the peculiar mode of secretion of the enamel precursor substances from and about projections from the ameloblasts; and 2) absent during the formation of the first and last layers of enamel (formed at the enamel-dentine junction and the true enamel surface respectively) by a given group of ameloblasts: hence there are no prisms in these regions. Abrupt changes in orientation of the mineralising front determine abrupt changes in crystallite orientation in the enamel (equivalent to the "prism-sheaths"of adult enamel). The secretory territories of individual ameloblasts are only equivalent to prisms in one particular pattern: one ameloblast may be related to more than one prism. Decussation of prisms is associated with the depressions in the mineralising front filling in from alternate sides in "zones". Zone formation begins as a spiral over cusp centres.

Light scattering from enamel depends on 1) the size; and 2) the orientation of its ultrastructural elements and 3) the wavelength of the incident radiation; blue light being scattered preferentially; hence the visibility of:- 1) the incremental striae; and 2) the decussating zones of prisms; and 3) the brown colour of the incremental striae when viewed by transmitted light.

The calcium content in developing enamel measured by the x-ray emission microanalytical method was found to increase steadily, from the surface of the developing enamel inwards.

"It is in the nature of an hypothesis, when once
"a man has conceived it, that it assimilates
"everything to itself,as proper nourishment;and,
"from the first moment of your begetting it, it
"generally grows the stronger by everything you
"see,hear,read,or understand. This is of great use."

The life and opinions of Tristam Shandy, Gentleman.

STERNE, 1759.

CONTENTS

I N T R O D U C T I O N

Before the commencement of the work described in this thesis, it was known that enamel consisted of myriads of uniformly-sized, fairly well orientated crystals of hydroxyapatite packed into an organic matrix of some nature. It was known* that a repetitive pattern of change in the orientation of its constituent elements was responsible, at least in part, for its division into so-called rod (or prism) and interrod (interprismatic) regions. It was known that the pattern of organisation of these larger components (i.e. the rods or prisms) varied greatly amongst the different mammalian groups. It had recently been demonstrated that the whole of enamel developed in an extracellular location (FEARNHEAD, 1960; WATSON, 1960).

This study examines the relationship between the cells which secrete enamel and the pattern of orientation of the elements in the formed tissue. A particular problem had been posed by the demonstration that the whole tissue developed in one site, i.e. in an extra-cellular location.(Previous explanations for the development of enamel structure had invoked a double siting, e.g. interrod substance from "terminal bars" and rod substance from ameloblast cytoplasm). Previous electron-microscopic studies of amelogenesis have been performed on rodent and primate teeth; presumably on the one hand because of the general availability of laboratory, rodent species; and on the other, because of the applicability of the results to man. Severe limitations are imposed by restricting the study to these two orders. I have studied developing material from members of the orders, Primates, Carnivora ,Ungulata,Rodentia, Proboscidea, Sirenia, Insectivora and Marsupialia.

The crystallite orientation patterns in developing enamel were studied in electron-micrographs of sections cut in many different planes. Confirmation of these findings was obtained from the study of stereo-pair electron micrographs of the ultra-thin sections, when

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* A more extensive (than Chapter 1) historical introduction to the subject of the structure and development of mammalian enamel has been prepared and is referred to in the text as BOYDE (1964 MS).

(continued overleaf).

the crystallite orientation could be visualised directly. The topography of the developing enamel surface was studied by two new techniques - by direct examination in a scanning electron microscope, and via the production of wax reconstructions from large-scale projection drawings made of the profile of the developing enamel surface in $\frac{1}{2}\,\mu$ thick sections of methacrylate embedded "electron-microscope blocks". The relationship of the ameloblasts and the prisms to the topography of the developing enamel surface has been studied in the electron micrographs of ultra-thin sections cut in different planes.

The main problem in studying enamel is that its structure must be seen in three dimensions - this is also the main problem in communicating the results. The photographs of the wax-reconstructions and models do not convey the same "information" as the originals. I have tried to overcome this problem by presenting one "stand-up" model to illustrate the convention adopted for representing the various surfaces of an imaginary block of tissue in the other illustrations (Appendix D).

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* My own perusal of the literature dealing with the structure and development of mammalian enamel did not reveal any up to date comment on the very early work in this field. An extensive review of this material was prepared, but it was felt that it would be out of place in this thesis, and it has consequently been bound as a separate volume (BOYDE, 1964 MS). Chapter 1 is a very short introduction to the development of knowledge regarding enamel: some more specific items dealing with the observations of earlier workers will be found in the context of discussion relating to them in the later chapters. Chapter 10 deals with the development of knowledge following the application of the electron-microscope to the study of enamel structure and development. It has been placed last so that this previous work can be discussed in the light of the present findings: the material which it contains follows on logically from the end of chapter 1.

FIGURE I

caption figure1 to face figure1 print on reverse of iv

Prism cross-sectional outlines.
The lines in this diagram represent (sectioned) boundary planes of abrupt change in crystallite orientation. There is only a gradual change in crystallite orientation between any two points which can be connected by a line which does not pass through such a boundary plane. Circumscribed regions in which crystallite orientation only changes gradually from one point to the next are referred to in the text as domains.

1) PATTERN 1. Cylindrical (circular in transverse section) prism boundaries with separate "interprismatic regions": found in Sirenia, Cheiroptera, Insectivora, and Odontoceti.

2) PATTERN 2. "Horseshoe" (cross-sectional shaped) prism boundaries:: prisms arranged in longitudinal rows with no regions which can be defined as interprismatic between prisms in the same row: found in Primate .
2A) same; but with a greater separation between the rows of prisms. Inter-row sheets can be defined: found in Ungulata, Marsupialia, and Lagomorpha.
2B) same; but the decussation of alternate (transverse) rows of prisms distorts the basic pattern (2): found in Myomorph and Scuiriomorph (Rodent) incisor inner-enamels. (Hystricomorph patterns are considered in section 2.4.3.2 and Fig. 2.23).

3) PATTERN 3. Arcade arrangement of prism cross sections.
The cervical, open side of the prism boundary faces a "gap" between two prism boundaries cervically. This means that there is no abrupt change in crystallite orientation from the centre of the prism to the narrow region situated between the two prisms on its cervical side - the "gap" is the "winged process"; there is no region which can be called interprismatic: found in human and elephant enamel.
3B) The prism boundaries are more complete,i.e. they extend through considerably more than a half circle as appreciated in transverse section. Pattern also found in man, and Carnivora.
3C) same; but with wider regions separating the boundary planes. It is now difficult to conceive of these regions as "winged processes" - one would call them "interprismatic", but they are still continuous with the regions "within the prisms" via the open side of the prism boundary. This pattern obviously approaches that called Pattern 1 above; found in Carnivora.

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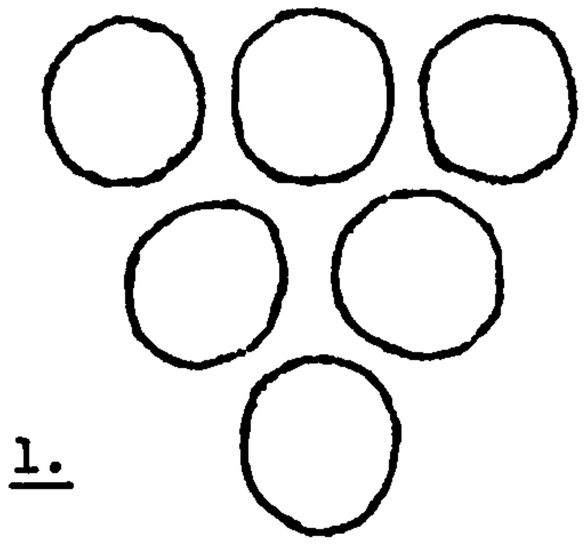
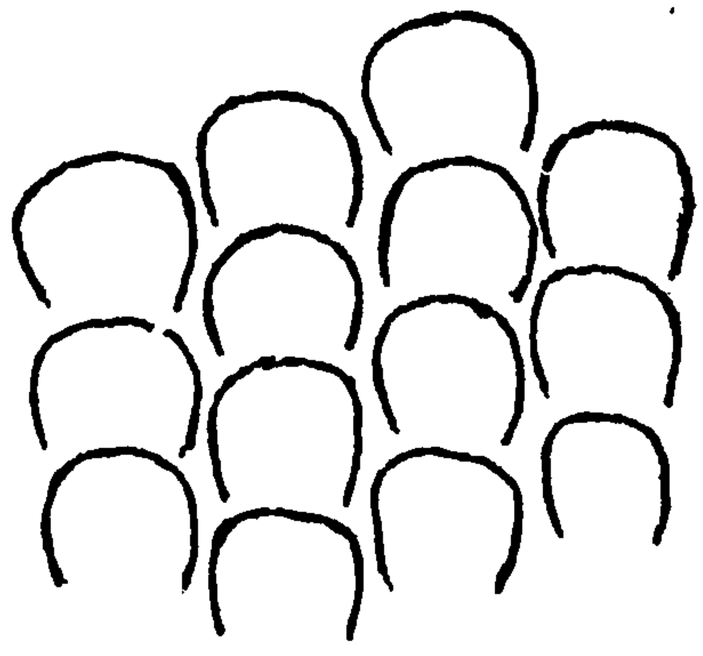
site.

FIGURE 1.



1.



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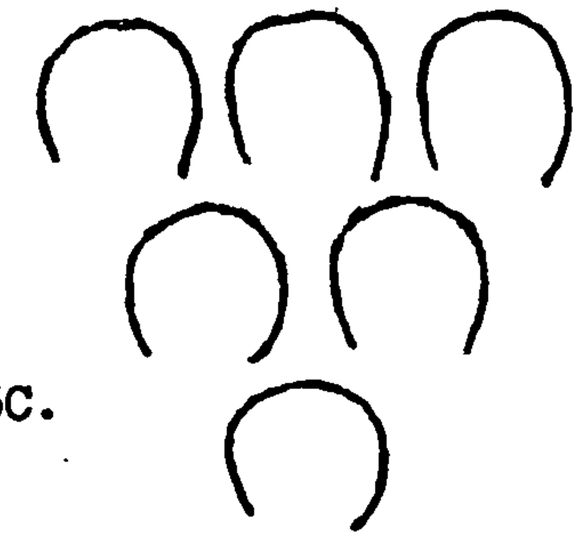
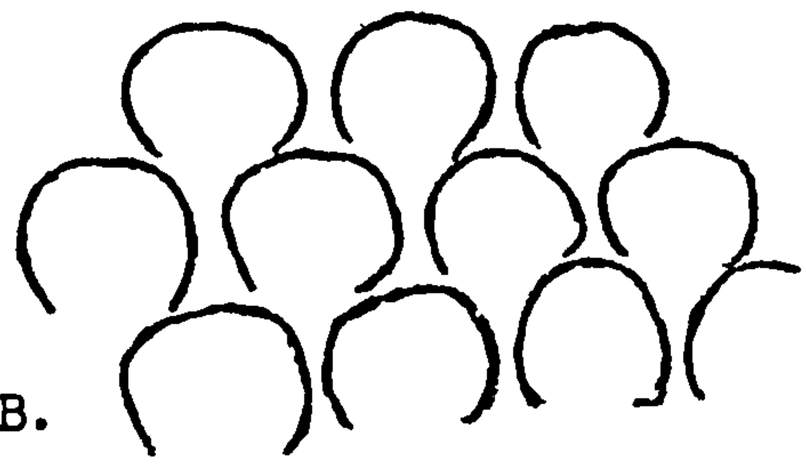
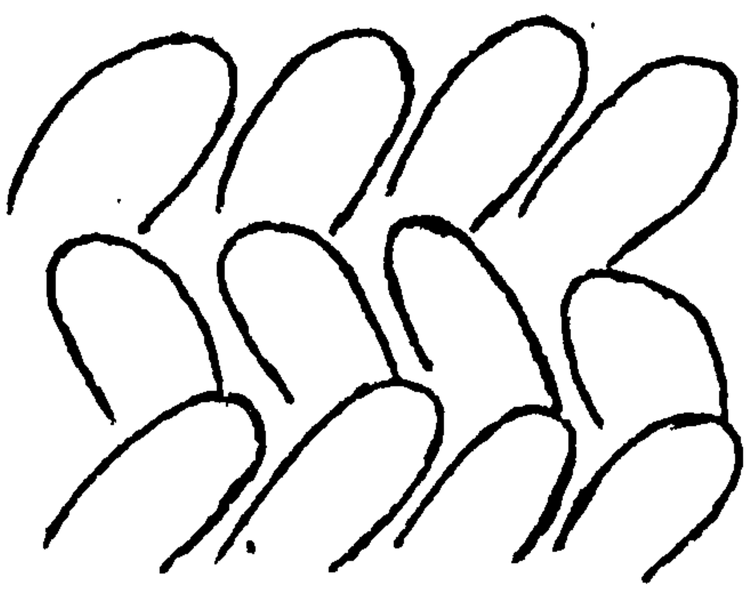


3C.

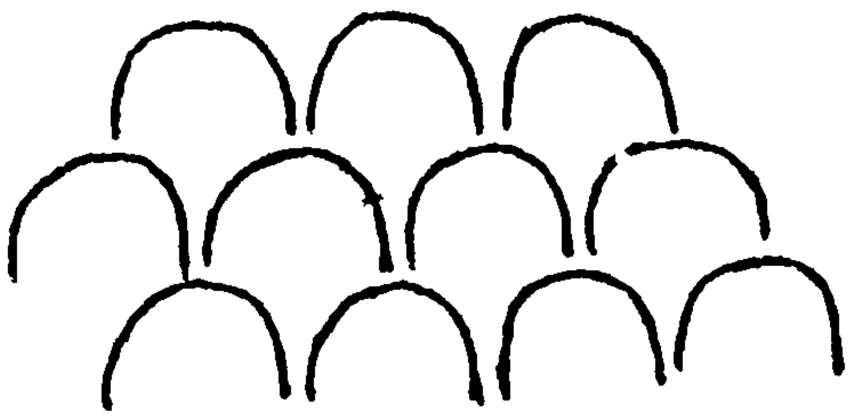
2A.



3B.



2B.



3.

The accompanying diagram (Figure 1.) shows many of the typical outlines of enamel prisms which have been encountered in this study. It belongs to every chapter, and it introduces the new nomenclature which is used in this thesis.

CHAPTER I

HISTORICAL INTRODUCTION

An almost explosive increase in the knowledge of visible enamel structure occurred simultaneously with a more general availability of good light microscopes in the years 1835-40. Most of that information which can be obtained with the ordinary light microscope was obtained during this period. It is more than difficult to establish priorities of discovery during this period, but PURKYNĚ and his pupil FRAENKEL (1835) seem to have been the first to publish an account of the microscopic anatomy of human teeth. The first account of a microscopic study of enamel development was published in the same year by RASCHKOW (1835), another one of PURKYNĚ'S pupils. The studies of MÜLLER (1837), RETZIUS (1837), LINDERER (1837) and NASMYTH (1839) were published shortly thereafter. It is evident that PURKYNĚ must be selected as the pioneer of this upsurge of interest in dental histology - FRAENKEL and RASCHKOW were his pupils and MÜLLER a fellow-professor: RETZIUS'S interest stemmed from his visiting PURKYNĚ in Breslau in 1835 (see COHEN (1940)), and NASMYTH'S interest from his translating these other workers. LINDERER, senior, (1837) laid claim to having discovered various features of enamel structure before PURKYNĚ and his followers, and there is no outstanding reason to doubt this claim. However, he certainly did not publish his discoveries first.

PURKYNĚ and FRAENKEL, LINDERER, RETZIUS and NASMYTH prepared specimens for microscopic observation by sawing sections from intact teeth. These sections were then thinned down and polished by rubbing them on the surface of fine abrasive stones. Dental Histology has not progressed much. The examination of such a "ground section" shows that the enamel is divided into a multitude of fine fibres which extend from the junction of enamel with dentine, towards the tooth surface. These fibres - the columns, Säulo, prisms rods, etc. of later authors - should not be confused with the "fibres" (Striae of HAVERS, 1689; filets of DE LA HIRE, 1699; "fasernstreifen" of SCHREGER, 1800) visible with the naked eye. (see 7.1). The relationship between these two sorts of "fibres" was established by PURKYNĚ

1.2 and FRAENKEL (1835, 1836) and LINDERER (1837). MÜLLER (1836) was the first to isolate (developing calf) enamel prisms. RETZIUS (NASMYTH, 1839 translation) was responsible for the invention of supplemental prisms. TOMES (1848) assumed that supplemental prisms must be present in order to account for the difference between the surface area of the enamel and the area of enamel-covered dentine. CZERMAK (1850), WILLIAMS (1886) and MUMMERY (1919) also believed in supplemental prisms. Measurements of the prism-repeat distance (diameter) at the enamel-dentine junction and the enamel surface show that there is no need to imagine that supplementary prisms exist. (PICKERILL, 1913; WILLIAMS, 1933 A; CHASE, 1924; HEUSER, 1956).

PURKYNĚ and FRAENKEL described the enamel fibres as having a quadrilateral cross-sectional shape due to the mode in which the fibres were packed together. RETZIUS (1837) thought the normal cross-sectional shape was hexagonal for the same reason - to him the name prism is due. LINDERER and LINDERER (1837) first figured the open-sided, horse-shoe shaped, cross section of the enamel prisms. Their figures show that the prisms are not closely packed together without an intervening substance and that the prism boundary is more often than not, incomplete. They also showed that the open-side of the prism boundary faces towards the cervical margin of the crown of the tooth, rather than towards the tip of the nearest cusp or incisal edge. NASMYTH (1839 - published in 1841) independently described this arrangement. (More specifically the arrangement dubbed "Anordnung I" by SMREKER (1905)).

PURKYNĚ and FRAENKEL (1835, 1836) and RETZIUS (1837) described a fine cross-striation of the enamel prisms recurring at intervals roughly equal to the width of the prisms themselves. LINDERER and LINDERER (1837) flatly denied the existence of these cross-striations as few have done since. PURKYNĚ and FRAENKEL, and RETZIUS also observed the effect of dilute acids on their ground sections, watching the process of dissolution on the microscope stage. They were able to see that a very fine "membrane" separated from the region of the prism-boundary (it should be noted that for these authors there was no inter-prismatic substance) and that this membranous prism-sheath (of current terminology) was divided by a series of more or less complete,

1.3 transverse partitions. They regarded the appearances seen in this simple experiment as indicating the existence of a more organic, less highly mineralised, prism-sheath material (interprismatic substance for these authors and many following), continuous with material of the same nature constituting septae within the prisms. They thus sowed the seeds of the idea that the cross-striations of the enamel prisms are related to a change in the mineral content of the enamel prisms along the length of the prisms. This idea is very widely accepted at the present day, as is also the existence of organic prism-sheaths. TOMES (1848) considered that the cross striations were "--- due to alternate dilatation and contraction of the fibres," ---.

PURKYNĚ and FRAENKEL (1835), RETZIUS (1837), and LINDERER and LINDERER (1837) described the plane of junction between enamel and dentine. The plane of junction as appreciated in a thin section presents what later authors have called a festooned or scalloped outline, with the points (headlands) between adjacent concavities (bays) pointing into the enamel. LINDERER and LINDERER (1837) noted that membranous (organic) extensions could be recovered from the position of "fissures" (running from the "Spitzen der Gruben" across one-third, - at the most one-half - of the thickness of the enamel) in ground sections demineralised with acid. These fissures are the tufts (possibly also lamellae) or current terminology. LINDERER and LINDERER (1837) distinguished between these Fissures (named Büschelfasern - i.e. tuft-fibres - by LINDERER junior himself in 1848) which they considered to be real structures, and cracks which might develop during the grinding of the section. PURKYNĚ and FRAENKEL, and LINDERER and LINDERER prepared ground sections of the inner one-third of enamel with the plane of section parallel to the enamel-dentine junction (tangential sections) and showed that these "Fissures" extended throughout the greater part of the length of the enamel as parallel wavy lines - in fact, as longitudinal "cracks" of some sort, and mostly confined to the inner third of the enamel. This arrangement of longitudinal tuft-rows was made much of by BEUST (e.g. 1924).

The clearly isolated projections from the enamel-dentine junction in human teeth, - more common in the cuspal or incisal enamel - and extending some 10 prism-widths (40) into the enamel at right angles to the enamel-dentine junction, were first described by RETZIUS (COHEN - 1940). These Spindles of modern terminology are in apparent continuity with the dentine and many contain apparent projections of the dentinal tubuli themselves. They remain attached to the dentine when the enamel is dissolved away with acid. TOMES (1848) and LINDERER (1848) also described the spindles (further details - Chap.9).

JOHN TOMES (1849) discovered that in the Marsupials, (with the one exception of the Wombat), Shrews, Hyrax and Jerboa, -" the greater number, if not all, of the dentinal tubes are continued into, and constitute a considerable portion of the enamel." In 1850, TOMES described the family-characteristic, enamel-fibre decussation patterns in rodent incisor enamel, and drew attention to the presence of cells or spaces - characterised by their distribution and isolation - in this tissue. (Other previous studies of the enamel tubules are considered in Chapter 9, and in more detail in BOYDE, 1964 MS).

RETZIUS (1837) is best known to Dental Histologists for his description of yet another variety of stripe or line in the "cortex striatus" (Blake, 1798, 1801), yet he was neither their discoverer, nor did he dwell much upon them. He called these lines "generally brown, parallel stripes", described their concentric disposition over whole cusps or incisal margins and concluded that they must reflect in some way the incremental mode of formation of the enamel (see 8.1.). In this description and conclusion he was anticipated by PURKYNE and FRAENKEL (1835). However, he made the important observation, since verified by a large number of authors, that these Brown Striae of RETZIUS correspond with the surface markings on the enamel called "kringsgewijze rimpels" (circularly disposed wrinkles) by LEEUWENHOEK (RETZIUS, 1837) and now known as Perikymata (PREISWERK, 1895, 1903) or the "Imbrication lines" of PICKERILL (1913).

CZERMAK (1800) pointed out that the circular wrinkles (Wulstchen) are absent from the surfaces of the deciduous human teeth.

LINDERER and LINDERER (1837) and VON BIBRA (1844) thought that the appearance of the circular wrinkles (Perikymata) on the surface of the enamel and the HUNTER-SCHREGER bands within, might be related. (The LINDERERS referred to the HUNTER-SCHREGER bands as "Querstreifen", in contrast to their "Schichtstreifen", i.e. the Striae of RETZIUS). VON BIBRA noted that the appearance he described of the HUNTER-SCHREGER bands "shining through" from beneath the surface, was very well marked in the teeth of a few large carnivores. CZERMAK (1850) pointed out the confusion between Perikymata ("wulstige Beschaffenheit") of the outer enamel surface and the HUNTER-SCHREGER bands shining through - that though they are parallel to each other they are distinct structural entities. CZERMAK wrote ... "As the peripheral ends of the prisms run straight to the surface, it is self-explanatory that this appearance can only be seen (shimmering) in the depth of the section in completed teeth, whereas it can be seen on the surface of incompletely developed teeth."

C.S. TOMES (1904, p.19) held that the Perikymata and the HUNTER-SCHREGER bands were interrelated. PICKERILL (1913) showed that the Striae of RETZIUS (and not the HUNTER-SCHREGER bands) "outcrop" at the "imbrication lines" (Perikymata). (Previous studies of the incremental lines are considered in more detail in 8.1.).

Longitudinal rows of prisms have been observed by a very few authors in passing comment (WILLIAMS (1896, A. p.478, fig.83 and caption), CARTER (1922, in marsupials) and WEIDENREICH figured such rows in the horse (1926, p.326, fig.23) and dog (p.328, fig.26). MUMMERY'S famous figure which purports to show the branching prisms in the wart-hog Phacocoerus (1919, p.54, fig.17 - also in various editions of WIDDOWSON, "Spec. or Dent. Anat." etc.) is, in fact, one of the best possible illustration of transversely sectioned longitudinal rows of prisms that has been published - it is the longitudinal rows of prisms that branch, not the prisms themselves). SHOBUSAWA (1952) recognised longitudinal row formation at its maximum development in the Ungulata and Macropodidae (marsupialia) (See figure 18 of MARCUS, 1931; and figures 2 and 3 of HÄUSELE, 1932). LUDWIG (1941) considered longitudinal rows of prisms as structural units - but his rows (Schmelzblätter) are several prisms wide - are in fact the prisms between adjacent "Schmelzsprünge" (the longitudinal "defects" appreciated in transverse

1.6 sections of the tooth as tufts; i.e. the longitudinal tuft-rows of BEUST; first described by LINDERER and LINDERER, 1837).

RUSHTON (1963) described a variety of enamel hypoplasia which is manifested in girls by a longitudinal, row-like, defective thickness of enamel. He considered whether this remarkable defect might be related to defective stem cells in the tooth germ giving rise to successive generations - ROWS- of ameloblasts with defective function.

HUNTER (1778) appreciated that enamel "contains animal mucilaginous matter". BERZELIUS (1813) estimated the organic content in enamel (by measuring the loss of weight on charring) to be not more than 2%; a value which is in good agreement - even though the experimental method is questionable (because water would be lost as well) - with currently accepted values.

HOPPE (1862) examined charred ground sections to study the distribution of organic matter in enamel. He found an even "greyness" in these preparations and concluded that that was an even distribution of the organic material. His analyses of the amount of organic material in enamel were performed principally on developing enamel (see later) and fossil material. He could not recover any remnants after the acid decalcification of adult enamels.

The further development of ideas concerning the organic content of enamel was associated with the use of a gold chloride "staining" technique (HEITZMANN, 1882; C.F.W. BÖDECKER (the elder), 1878· SUDDUTH, 1886; ABBOTT, 1880, 1889).

C.F.W. BÖDECKER'S son, C.F. BÖDECKER, developed a careful decalcification technique (in a thick celloidin solution). He named the lamellae (9.3) the leaf-like processes of "organic" substance running longitudinally and extending from the enamel-dentine junction to the enamel cuticle (BODECKER; 1909, and previously). He also described the tufts (9.3) and separate, organic prism-sheaths. The cross-striations of the enamel prisms were, for him, built of extensions of the organic prism-sheaths. In collaboration with GIES (BODECKER and GIES 1924; GIES 1924-26) he performed histochemical tests to establish the protein content of his decalcified enamel remnants. In 1924-26 he produced for the first time his sponge (organic matrix) - stone (mineral content) analogy of enamel composition.

1.7. WILLIAMS (1896) denied the presence of any organic material in enamel and held his opponent's views to be "vain imaginings." TOMES (1896) concluded that "there is very little organic matter in enamel, not enough for quantitative estimation." BLACK's (1916) was probably the last voice of authority to be raised against the existence of organic material in enamel.

STACK (1955) stated that "the most important organic component (of enamel) is thought to be a keratin, as suggested by HOPPE more than a century ago." THOMPSON (1886) considered "that the salts of time are deposited -- in an organic matrix of horny matter - keratine." STACK'S (1955) own findings that enamel contains a eukeratin seem to summarise the view of that period. PIEZ (1960) and EASTOE (1963 - personal communication) have both said that the enamel protein is unlike any other known protein. An outstanding recent contribution by EASTOE (1963), and the excellent book of LEICESTER (1949), deal with the history and details of this aspect of enamel structure in praiseworthy detail.

NASMYTH (1839 B) described a fine membrane which could be isolated by acid from the surface of an unerupted or newly erupted tooth, and which he called the "persistent dental capsule". He considered this layer to be continuous with the cementum of the root, and equivalent to the cementum covering the coronal enamel in herbivores. J. TOMES (1856) and C.S. TOMES (1872) concurred in the opinion that "NASMYTH'S membrane" was cement in spite of the fact that C.S.TOMES noted that it "gives off a smell like burning horn " when heated. PAUL (1894) used his "discovery of horny, or calcified, flattened epithelium between the enamel and cementum in herbivorous teeth" to clinch his case that "NASMYTH's membrane" was not cementum, but was of epithelial origin. PAUL (1896) described his membrane as consisting of two layers, - an outer layer of large, flattened epithelial cell and an inner, thin, transparent pellicle, usually marked with hexagonal impressions derived from the ends of the enamel prisms. The isolation of "NASMYTH'S membrane" was first described by RASCHKOW (1835), a fact which was acknowledged by NASMYTH (1839 A. p.154) himself in his own published translation of RASCHKOW. RASCHKOW also described the acid-isolated membrane as being continuous with the cementum of the root.

1.8 The continuity of "NASMYTH'S membrane" and the lamellae and "organic prism sheaths" has been confirmed by all workers who have dwelt in this field. The most commonly accepted view of the development of the two components (FRANK, 1949 only recognised a "cuticule unique." NASMYTH'S own membrane (1839 B, p.313) consisted of two layers) of NASMYTH'S membrane is that of RÖSE (1839), CHASE (1926), ORBAN (1926) and others (before and since) that the ameloblasts "secrete" a final cuticular produce which persists as the primary enamel cuticle, and are themselves transformed (with other cell layers) into the secondary enamel cuticle (reduced enamel epithelium of other authors) by a process of incomplete keratinization. The primary enamel cuticle (for which I shall use the term enamel cuticle) is approximately 1μ in thickness in most species and is an organic membrane of relatively high mass (GREULICH, 1956). When isolated from the surface of the tooth by acid it shows impressions reminiscent of the ends of the enamel-prisms.

CHASE (1926) thought that the "rod-end markings" on the enamel cuticle were produced by remains of ameloblasts on the one side, and detached portions of enamel (prism-sheaths) on the other. CHASE also considered that the outer cellular portion (reduced enamel epithelium) of the NASMYTH'S membrane contained components derived from all the cell layers of the enamel organ except the stellate reticulum. PANNESE (1961) has shown that the stellate reticulum cells are involved, at least in the enamel organ of the cat.

VON BRUNN (1887) studied the enamel-free areas of the molars of the rat and showed that all the enamel-organ cell-layers underwent a metamorphosis to form a "reduced enamel epithelium" over these areas, like the rest of the enamel-organ did after it had completed the function of depositing enamel.

CAUSH (1905) stained whole teeth by immersing them in alcoholic fuchsin solutions for long periods before preparing ground sections. He noted that the stain penetrated the enamel spindles and tufts,, and also into some vaguely defined interprismatic (? prism-sheath) location. BEUST (1912) repeated these experiments, but confined the access of the dye to the pulp chamber of the teeth. He also noted penetration of the fuchsin into the lamellae, tufts, spindles and "interprismatic"

1.9 substance. These results have been confirmed many times since and also using aqueous dyes in living dogs by FISH (1926 and a series of publications until 1932), who introduced solid methyl blue into the dental pulp; by BERGGRÉN (1947, review) who injected methylene blue in the "vestibulum of molar region" of live dogs; and by FRANK (1950) who injected indigo-carmine and methylene blue into the sub-mandibular lymph nodes of human and canine corpses. All these workers noted that the dye was confined to <u>separate</u> prism-sheaths; they thus provided a more accurate description of its location and provided more evidence for the already strong concept of the prism sheath as a more organic, less calcified and therefore, more permeable structure.

KANNER (1938) demonstrated a centripetal permeability (i.e. from the tooth surface inwards) of enamel of adult human teeth in situ by applying a methylene blue paste to the tooth surface some 5-30 mins. before extraction. JANSEN and VISSER (1950) used a fluorescent dye to demonstrate centripetal permeability of dog enamel.

BUNTING and RICKERT (1918) demonstrated the osmotic flow of Na^+ and Cl^- ions through the enamel of extracted teeth. The permeability of enamel to ions has since been demonstrated many times using radio-isotope labelling techniques. (For example, WASSERMAN, BLAYNEY, GROETZINGER and DE WITT (1941) - WAINWRIGHT and LEMOINE (1950) and SOGNNAES, SHAW and BOGOROCH (1955)). AMBERSON (1927), and KLEIN and AMBERSON (1929) demonstrated a dialytic flow of Na^+ and Cl^- ions through the enamel of extracted teeth, thus showing that enamel is a "permeable membrane".

SPRAWSON (1930) in <u>Macropus ualabatus</u>, and McCREA and ROBINSON (1935-36) in <u>Macropus rufus</u>, demonstrated the continuity of the dentinal and enamel tubules in marsupial enamel, as also the patency and permeability of the enamel tubules, using dye diffusion experiments in which the access of the dye to the dental tissues was confined to the pulp-chamber. (My own similar experiments are reported in Chap. 5.10 - discussion in 9.1.).

LEHNER and PLENK (1936) reviewed the literature on the question of enamel permeability in their accustomed thorough fashion.

1.10 It is now generally accepted that the mineral component of mammalian dental enamel belongs to the apatite system. That it is a calcium-phosphate was appreciated by VON BIBRA (1844) and its apatitic nature was seriously considered by HOPPE (1862). Confirmation of the apatitic structure came first from the x-ray diffraction studies of FUNAOKA (1926) and GROSS (1926).

VON BIBRA used the Prussian Blue ($K_4Fe(CN)_6$ after HCl treatment) test for iron in a rodent incisor section which had been heated to red heat, in which he noted that the red-brown colour of the surface enamel layer was retained. DAM and GRANADOS (1945) also concluded that "inorganic iron alone accounts for the total colour of the pigment".

An account of the early stages of development of the tooth germ will be found in the standard texts, e.g. ORBAN (1957), SCOTT and SYMONS (1958). Descriptions of the behaviour of the inner enamel-epithelia, cell nuclei and cytoplasmic inclusions just before and during the onset of dentinogenesis, vary with the species examined and the author.

The shape of the amelo-dentinal junction is determined by the shape of the plane of contact between the odontoblasts and the presumptive ameloblasts. (RASCHKOW 1835; HENLE, 1841; RAINEY, 1859; ERDHEIM, cited by GOTTLIEB, 1920; ORBAN, 1926.. Many other workers e.g. WEDL (1872) had thought that the festooned shape of the enamel-dentine junction was occasioned by a dentine-resorbing activity of the inner enamel epithelium before it took on its role of enamel formation). This shape is <u>fixed</u> once the first layer of dentine has been laid down and mineralized - this activity is appreciated in decalcified (light-microscope) histological material, as an increase in the width of the clear "basement-membrane" separating the odontoblasts and presumptive ameloblasts. Dentine formation always precedes enamel formation in all mammals; this has only be doubted by HOPEWELL-SMITH and TIMS (1911) in the case of the Wallaby, <u>Macropus billardieri</u>. These authors thought that enamel formation may precede dentine formation "in some cases".

All authors are agreed that the nuclei of the internal enamel epithelial cells have moved towards the end of the cell away from the

1.11 newly formed dentine (outer end), before these cells commence to secrete enamel. The Golgi apparatus (JASSWOIN, 1924; BEAMS and KING, 1933) and the Centrosomes (de RENYI, 1933) are situated on the side of the presumptive ameloblast nucleus towards the stratum intermedium, but they acquire a position on the inner side of the nucleus during the final migration of the nucleus.

The stratum intermedium ends of the columnar internal enamel epithelium cells are attached to those cells by intercellular bridges (ANNELL 1882) and to each other by Outer terminal bars. Inner terminal bars develop at the dentinal ends of the pre-ameloblasts during the final stages of the reversal of polarity - a term used to signify the movement of the Golgi apparatus, centrosomes and nucleus - which is widely held to be associated with a change in the functional polarity of these cells (WILLIAMS, 1895, 1896, 1897; COHN, 1897; LAMS, 1920, 1921; HELD, 1926; CHASE, 1932; ESCHLER, 1938; KEREBEL and GRIMBERT, 1958). The Mitochondria of the pre-ameloblasts also move to acquire a position on the stratum intermedium side of the nucleus - before this they are apparently scattered throughout the cytoplasm. Most previous workers have assumed that one ameloblasts is responsible for the formation of one enamel prism. KITCHIN (1933), WASSERMANN (1943), GOTTLIEB (1943), and AGNEW (1947) dissented from this opinion .

The older theories of enamel development differ only with respect to the exact mode of origin of the prism from the ameloblast; for example, whether the ameloblast is itself transformed into the prism or whether each ameloblast "secretes" a prism, the secretion from each ameloblast remaining separate for some reason.

The layer of ameloblasts was called membrana adamantinae by PURKYNĚ and RASCHKOW (1885). NASMYTH (1839 A. p.136) translated these authors - "Each of its perpendicular fibres is to be regarded as an excretory organ or gland, destined for the secretion of the enamel fibre corresponding to it." (my italics). Of the development of the cross-striations of the enamel prisms, RASCHKOW wrote only that each fibre "is composed of layers disposed in a transverse direction". RASCHKOW's membrana praeformativa was the basement membrane between the inner enamel epithelium and the dental papilla. HUXLEY (1835) fell into the serious error of identifying it with the forming surface layer

1.12 of developing enamel. This interpretation did a great deal to confuse and hinder thought on enamel development for a long time afterwards.

TOMES (1848) subscribed to the view of SCHWANN (1839)translated 1851) that a transformation of successive layers of the enamel forming cells occurred - he saw the different layers of the enamel organ as representing stages in the transformation of the cells into units which would end up as the cross-striations of the enamel prisms. Presumably, like the other authors who adopted this view, (HENLE, 1841; KOELLIKER, 1854; WALDEYER, 1865; HERTZ, 1866) he thought that enamel formation ceased when there were no more layers of enamel organ cells to be transformed, for he makes no mention of cell division. MARCUSEN (1853) and KOELLIKER, (1854) thought that the "enamel cells become, by their complete ossification, the enamel-fibres."

HUXLEY'S (1853) incorrect identification of the site of RASCHKOW'S membrana praeformativa, meant that he had to adopt the view that "Neither the capsule (dental follicle) nor the enamel-organ take any direct share in the development of the dental tissues, all three of which - viz. enamel, dentine and cement - are formed beneath the membrana preformativa, or basement membrane of the pulp." LENT (1855) agreed with HUXLEY'S identification of the membrana preformativa, and also agreed that its presence implied that the ameloblasts could not be directly transformed into prisms. LENT, however, thought that the enamel was secreted or excreted through this membrana preformativa, the secretion of each cell in some way retaining its individuality. ROBIN and MAGITOT (ca. 1859 transl. 1863) agreed with the view of LENT and added, as evidence for the secretory view of enamel formation, their observation that they could find - "no trace of super-position of layers of cells" in fragments of prisms longer than ameloblasts.

TOMES (1856 p.216) looked at HUXLEY'S membrana preformativa material on edge and saw - "that the enamel fibres pass through to the surface of the apparent membrane." RÖSE (1891) throught that the proponents of the theories of Secretion (PURKYNĚ and RASCHKOW, 1835; LENT, 1855; ROBIN and MAGITOT, 1863, KOLLMAN 1870; BAUME, 1882; WILLIAMS, 1884, 1886; VON SPEE, 1887; ANDREWS, 1891; and others) and Metamorphosis (or transformation of the ameloblast - SCHWANN, 1839;

L.13. HENLE, 1841; TOMES,1848; MARCUSEN,1853; KOELLIKER,1854; HERTZ,1866; WALDEYER,1865,1870;SUDDUTH,1884; ABBOTT,1889) were not discussing the major issue, which was for him ...."whether the same cells serve the purpose of forming the hard tissues from the beginning to the end, or whether there are always more cells replacing those which have been completely metamorphosed into the rudiments of these tissues.

The question of whether a separate interprismatic substance really exists has been argued by many workers since CZERMAK (1850)described a special intermediate substance ("besondere Zwischensubstanz") between adjacent prisms. Very few workers have made it clear as to what they have regarded as interprismatic regions in the light-microscope image.Naturally enough, the similarity in the situation of the interameloblastic and interprismatic "substances" provided sufficient evidence for many workers to accept the origin of the latter from the former (UNDERWOOD and WELLINGS, 1913; MEYER, 1925, 1935; GÖLLNER, 1930; GYSI, 1931). BROOMELL (1912) believed that the walls of the ameloblasts became the interprismatic substance and that their bodies became the prisms. BAUMGARTNER,(1911) argued that there could be no interprismatic substance, because there were, for him, no interameloblastic spaces. ANDRESEN (1902) held that the central part of the prism developed by the transformation of TOMES' process, whereas the peripheral part and the interprismatic substance developed in a secretion from the ameloblasts. STUDNICKA (1917) considered that the forming enamel was separated from the ameloblasts by a membrana preformativa composed of fine fibrils from the ameloblasts - and thus denied any relation between the ameloblasts and the prisms - he mentioned "self-formative properties in the enamel layer".

COHN (1897) demonstrated a terminal bar apparatus at the dentinal ends of the ameloblasts - in, of course, an intercellular position. This did not deter the majority of adherents of the view of the intercellular origin of the interprismatic substance : they either simply confined their interests to the region below the terminal bars, assuming an intercellular origin of the interprismatic substance;

and an intracellular origin of the prisms (ie. transformation of the ameloblasts into prisms, e.g. CHASE, 1932) or they took no notice of the terminal bars whatever (e.g. NUCKOLLS, SAUNDERS and FRISBIE, 1943, A and B; NUCKOLLS, LEICESTER and DIENSTEIN, 1947; SAUNDERS, NUCKOLLS and FRISBIE, 1942 denied their existence.) or assumed that the terminal bars played no part in closing the intercellular gap between the ameloblasts, so that material could pass freely from an intercellular to an interprismatic position in spite of their existence (MEYER, 1925, 1935; ORBAN, 1925 B, GÖLLNER, 1930). HATTYASY (1938) believed that the terminal bar apparatus gave rise to separate, cylindrical projections which functioned as preformed prism-sheaths into, and between, which the enamel was secreted. LAMS (1920, 1921), ORBAN, (1926 A), RYGGE (1934), ORBAN, SICHER and WEINMANN (1943) and MARSLAND (1951) commented on the apparent continuity of the terminal bar apparatus and the interprismatic substance and assumed the derivation of the interprismatic substance from the terminal bar apparatus. This view is the one accepted by the authors of our principal undergraduate textbooks (e.g. SCOTT and SYMONS (1958, p.133) and ORBAN (1957, p.99).

A few authors have attempted to identify a special type of cell in the ameloblast layer to which SAUNDERS, NUCKOLLS and FRISBIE (1942), who studied the rat molar, gave the name Kionoblast. These special cells were supposed to give rise to an organic dentino-enamel membrane separating the enamel and dentine, and also to the spindles and tufts. SYMONS (1955) identified these same cells in the rat molar and incisor ameloblast layers and thought that they formed the inter-prismatic substance. KEREBEL and GRIMBERT (1958 and 1961) concluded that the Kionoblasts are artefacts and/or degenerating cells which would confirm the opinion of RUDAS (1906, cited by LEHNER and PLENK, 1936) that degenerating cells can be found in the ameloblast layer during its "formative stage".

Secretory Granules in the ameloblast have been described innumerable times. ANNELL (1882: cited by LEHNER and PLENK 1936), and VON SPEE (1887) noted the similarity of the staining reaction of the material contained within these secretory granules or vacuoles,

1.15 to that of the newly deposited enamel "matrix". VON SPEE (1887) stained developing enamel with OsO_4 (incidentally differentiating certain cells of the ameloblast layer with a description which would warrant their identification as kionoblasts). KEREBEL and GRIMBERT (1958) are among the latest authors to show that the - "Products of elaboration seen in the apical cytoplasm or in TOMES' process have the same tinctorial affinities as enamel matrix." HAMPP (1940) studied the mineral content of the secretory ameloblasts by microincineration techniques, and found them to contain large quantities of calcium and magnesium (ash), more especially concentrated towards both ends of these cells.

Among the more unfortunate identifications of the nature of the secretory material contained in these droplets are those of ORBAN (1926 A) who thought it was fat, and of SAUNDERS, NUCKOLLS and FRISBIE (1942) who thought it was eleidin - the name given to a precursor of keratin in epithelial cells. VON SPEE (1887) showed that the osmiophilia of his "droplets" was not due to a lipid content because it was not removed by a prior treatment with ether. ANDREWS (1891, 1893, 1900) identified the secretory granules with the "calcospherites" of HARTING (1872) and RAINEY (1857, 1858, 1861), an occasion perhaps never equalled in dental histology for its unfortunate consequences. HARTING (1872) had made it absolutely clear that calcium phosphate "calcospherites" never formed - the information available to ANDREWS at the time that he identified the "misnamed granules" as calcospherites, must certainly have been in favour of a predominant calcium phosphate composition of the enamel mineral. Naturally ANDREWS also identified the acid-resistent organic matrix of the first formed enamel, i.e. the material of HUXLEY'S (1853) membrana preformativa, with the calcoglobulin of HARTING. WILLIAMS (1895 and 1896 A) seems to have leaned slightly to ANDREWS view, but in 1987 he only accepted - "with reserve the experiments of RAINEY, HARTING and ORD". However, at a later date he (WILLIAMS, 1923 B) wrote of "calcospherites of which the sections or varicosities of the rods are built up", thus finally placing his influential opinion on theside of MUMMERY (1914 A, 1915, 1919). MUMMERY attempted to overcome the objection that there is very little carbonate in enamel by proposing that the enamel mineral was initially deposited as calcospherites of calcium carbonate, which later

1.16 disintegrated "to form the finally consolidated enamel" when phosphates were "added from the blood".

Calcification of dentine starts from isolated points in the newly deposited organic matrix and concentric layers about a given point calcify in turn, so that spherical zones of mineralized matrix are formed - these zones were also identified with the "calcospherites". Where these dentinal "calcospherites" do not fuse, poorly mineralised "interglobular dentine" remains. UNDERWOOD (1898) published pictures purporting to show "interglobular" spaces in enamel.

The most commonly employed histological material for the study of the development of enamel has long been the rodent incisor, simply because of the availability of the animals and the fact that these teeth are always growing. Discussion in the dental literature of "little piles of calcospherites" (MUMMERY, 1919) fusing together to form the enamel rods, and the elimination of comparative dental histology from the american and german dental school curricula, were probably chiefly responsible for the often repeated, erroneous identification of rows of transversely-sectioned prisms with rows of calcospherites and /or transverse striations of the prisms Many ridiculous situations have evolved where the rat has been used as a laboratory animal and the decussation pattern (see 2.4.3.2.) of the inner enamel rods has not been recognised. CHASE used many experimental procedures to test "possible factors in the genesis of transverse prism stripes" in the rat incisor, including altered dietary rhythm, altered "light and darkness" periods, disturbed rhythms of activity and rest and intermittent water deprivation (CHASE and WERLE, 1935 - 36 A,B ; CHASE, 1937 A,B) - all, of course, without any effect on the organisation of the prism rows in the inner-enamel.

Development of Tufts and Lamellae. The longitudinal disposition of the tufts and lamellae has long suggested that they may arise as cracks at some stage in development. Opinions vary in connection with the exact time of origin, and as to whether these structures may remain latent or masked, in the sense of their being weak points in the structures which are only revealed as the result of a traumatic event, (GRÄFF , 1921; AKAMATSU, 1928; WHITE, 1931).

1.17 LINDERER and LINDERER (1837) referred to the tufts as longitudinal "fissures", which could be distinguished from ordinary cracks because an organic residue could be isolated from them on decalcifying a ground section. SOGNNAES (1950) noted that "organic membranes can be preserved from any surface of enamel where fixative and acid have good access", and considered this observation to be highly relevant to the question of the origin of lamellae. Similar observations had been reported by TOMES (1856, Footnote on p.217) and MILLER (1902, Fig. 28). FRISBIE, NUCKOLLS and SAUNDERS (1944) studied the penetration of coloured fixative solutions into the enamel and noted a rapid, premature spread along lamellae (?cracks).

GOTTLIEB (1915) was one of a school of thought which held that both the tufts and lamellae have a real dentinal component. ORBAN (1926 B) felt that only the lamellae have a dentinal component. Others have held, either blankly that tufts and lamellae have no dentinal component or, that their extension into the dentine is proof positive of their artefactual origin in these cases - I can only identify myself with the latter opinion.

An origin from special cells in the ameloblast layer (Kionoblasts) has been mooted (SAUNDERS, NUCKOLLS and FRISBIE, 1942) and their origin from dentine by WEIDENREICH (1926) and others.

Structure of Tufts and Lamellae. There is still no general agreement on the elements involved in the formation of the tufts and lamellae, viz. whether they are prism- or interprismatic-substance or both. (Bibliography in ORBAN, 1926, 1957; GUSTAFSON, 1945). It is however, common ground that there is some imperfection in calcification in these regions, associated with the retention of more organic material. (The tufts are regarded as interprismatic cracks by SCHMIDT, 1963). The name "tufts"(given to the appearance in transverse sections) conjures up a picture of a lot of separate fibres separating from a common stalk. This is a false picture, caused by the projection of the images of different parts of one "wavy" structure (a plane of poorly calcified enamel components) into one plane. (ORBAN, 1926; MEYER, 1935).

GUSTAFSON and KLING (1948) noted a correspondence between the longitudinal tuft rows and variations in microhardness measurements,

1.18 as a result of which they suggested the name "longitudinal soft layers" for the tufts.

RÖCKERT (1955) found, as a result of microradiographic studies, that "tufts appear to contain about as much calcium as dentine." BAUD and HELD (1956) correlated the low x-ray absorption with an increase in the silver "staining" of the tufts. DARLING and CRABB (1956) have also used microradiography to establish that the tufts are hypocalcified. The literature on the question of the origin and structure of tufts and lamellae is truly prolific. (Bibliographies in MEYER, 1925; FABER, 1928; GUSTAFSON, 1945; KEREBEL and GRIMBERT 1958; and KLEES and BRABANT, 1959).

The Ultrastructure of Enamel. The most important advances in the knowledge of enamel structure followed the application of the methods of polarisation microscopy and x-ray diffraction. This allowed the deduction of the apatitic nature of the enamel mineral to be made (HOPPE, 1862; SCHMIDT, 1924; GROSS, 1926; FUNAOKA, 1926), and information to be obtained about the orientation of the crystalline substructure with respect to morphological features of the enamel, for example, its surface, or the prism direction (VON EBNER, 1906; SCHMIDT, 1924, 1937 A and B, 1963; CAPE and KITCHIN, 1930; KITCHIN, 1933; KEIL, 1936; THEWLIS, 1932, 1934, 1937, 1939; BALE and HODGE, 1936; WYART and TOURNAY, 1949; LYON and DARLING, 1953, 1957; SCHMIDT and KEIL, 1958; POOLE and BROOKS, 1961).

Polarised Light Microscopy . The fact that enamel is birefringent was reported by VALENTIN (1861) and HOPPE (1862). HOPPE concluded that the birefringence of enamel indicated a crystalline structure. VON EBNER (1890) disagreed with this conclusion (details in BOYDE, 1964, MS).

VON EBNER (1906, p.137) noted that the prisms, when observed in the polarising microscope between crossed-nicols, were never completely "neutral" in any position - "this observation is of importance because it shows that in no case do the prisms consist of single crystals". He determined that the optic axis of the birefringent material in enamel did not coincide with that of the prisms,

1.19 but diverged - by as much as 20° in occlusal enamel, and 6° - 7° near the cervical margin. He showed that the divergence in the lateral enamel" (POOLE and BROOKS, 1961) was towards the cervical margin (i.e. the outer ends of the ordered elements towards the cervical margin).

SCHMIDT (1924) proposed (though can hardly be said to have proved, as he claimed in 1963) that the crystals in enamel were apatite.

CAPE and KITCHIN (1930) recognised the existence of two predominating and different crystal directions, corresponding to the prism - and interprismatic-substances respectively.

KITCHIN (1933) failed to recognised the decussation pattern of rat incisor enamel and came to the conclusion that the crystallites were arranged in the enamel prisms with their c-axes lying roughly at right angles to the length of the prisms.

KEIL (1936) determined the orientation of the crystallites in the interprismatic substance - in ground sections of human enamel where the prisms ran parallel with the plane of the section - to be up to 40° from the prism axis.

LYON and DARLING (1953) examined human dental enamel by polarised light and found a cervical deviation of the crystallites from the prism axes of 0-44°. The same authors later (1957) found a mean deviation (outer pole of the crystallites cervical) of 18° ± 7° from the prism axis.

X-ray Diffraction. GROSS (1926) and FUNAOKA (1926) obtained arced x-ray diffraction patterns from adult human enamel, resembling those of the mineral apatite, and can thus be said to have been the first to come close to proving the apatitic structure of the enamel mineral (as well as confirming its preferred orientation).

CAPE and KITCHIN (1930) found a marked degrèe of preferred orientation of apatite crystallites in enamel from their "rotating Roentgen ray photographs". THEWLIS (1932, 1934, 1937) repeated these observations on longitudinal sections of teeth and determined the orientation of the hexagonal axes of the apatite crystallites with respect to the surface of the enamel.

1.20 THEWLIS (1939), after further x-ray diffraction studies, related the crystallite (fibre) axes to the prism axes. He found two groups of crystallites, in longitudinal sections of human enamel, the one making an angle of 5^{o}, and the other 40^{o} with the prism axis (both deviating cervically). THEWLIS (1939) did not think that these two groups coincided with the prism- and interprismatic-substances, but in 1940, he considered that - "when the x-ray results are combined with the results of examination by the polarising microscope -- the conclusion arrived at is that whereas the prism material is characterised in general by a predominance of the 5^{o} group of crystallites, in the interprismatic substance the 40^{o} group usually predominates."

SCHMIDT (1937, A) proposed that the double fibre structure observed by THEWLIS (1934, 1937) was due to the decussation of the prisms themselves. SCHMIDT held that the crystallites were parallel with the prisms themselves. SCHMIDT'S proposal ignored the fact that THEWLIS had observed this divergence on either side of the normal to the surface of the tooth in a longitudinal section - SCHMIDT'S effect might only be noticed in transverse or oblique sections. However, in a paper published shortly thereafter, SCHMIDT (1937, B) reported a polarised-light study of the orientation of the crystallites in single prisms of developing Elephas indicus enamel. He recorded a feather-like orientation pattern with the crystallites diverging either side of the central (parallel) axis of the prisms, with an angle of 15-20^{o} between the extinction directions in the two halves of the prism. He then (1937, B) considered this pattern to be the explanation of THEWLIS'S (1934, 1937) double fibre-axis.

WYART and TOURNAY (1949) concluded from their x-ray diffraction studies that the axes of the crystallites were contained with a cone of semi-angle 30^{o}; the cone making an angle of 10^{o} with the surface of the tooth, the outer end inclined occlusally. Their polarisation microscopy results confirmed this observation, and extended it to include two groups of prisms - in alternate HUNTER-SCHREGER bands - which made an angle of up to 30^{o}, either side of the normal to the surface of a transverse section.

POOLE and BROOKS (1961) using the polarising microscope and x-ray diffraction techniques - concluded that the crystallites in

1.21 human enamel diverged from the axis of the prisms on every side, but that a greater proportion diverged (outer poles) towards the cervical margin. They made no provision or explanation for the interprismatic substance.

Electron Microscopy. The crystallite orientation pattern proposed by POOLE and BROOKS (1961) is very similar to the model proposed by HELMCKE (1963, 1958, etc.) as a result of his studies of replicas of fractured enamel surfaces in the electron microscope. These two models (POOLE and BROOKS, 1961; HELMCKE, 1958, etc.) were the most comprehensive to have been proposed before the commencement of the work described in this thesis. They correspond rather closely to my PATTERN 3 arrangement (Figs. 1.3., 2.13.3).

Other previous electron microscopic studies of enamel structure and development will be considered in Chapter 10 - after the Chapters on my own observations - in order to avoid unnecessary repetition - This makes it possible to discuss these works in comparison with the present report, but they are also to be treated as an introduction to the following Chapters (2-9 inclusive).

Enamel Development (Personal Observations)

2.1. Introduction. FEARNHEAD (1961 B) stated :-"Crystals in the enamel rods, normally have a different orientation from those in the inter-rod region. One of the most important details of amelogenesis which needs to be established with certainty is how this difference in orientation is brought about." This has been much the problem which interested me. First - What is the repetitive pattern of crystallite orientation responsible for the division of mammalian dental enamel into the light-microscopically visible repetitive structural units, the prisms? Second - How is this pattern related to the individual formative cells, the ameloblasts? Third - Can the various shapes of, and the patterns made by, the prisms themselves in different mammalian enamels be comprehended as only small variations on a general theme of enamel development in the mammalia? Fourth - What is the nature of the cross-striations of the prisms? These were the major points in mind when I embarked on a comparative study of amelogenesis in mammals.

The work of HELMCKE (1953-60) would have suggested that there was no doubt of the existence of a repetitive pattern of crystallite orientation in the enamel (in fact this is the only feature seen in replicas of fractured enamel surfaces). However, this was only one set of results from one method, and a great majority of opinion held that nearly every enamel structure - e.g. Prisms, Prism-Sheaths, Interprismatic Substance, Cross-Striations - depended for its existence on some variation in its organic content from an adjacent structure.

The accepted view of enamel development at the commencement of this study was that the ameloblasts themselves were converted into prisms, and the terminal bar apparatus into the interprismatic substance. FEARNHEAD (1960), had shown that enamel develops in a secretion of the ameloblasts, and he identified the terminal bar apparatus with accumulations of extracellular granules -

2. a fibre-precursor substance - in an inter--TOMES'-process, and hence interprismatic, position.

The only convincing explanations of the development of the cross-striations of the enamel prisms which had been put forward had been those of Anna-Greta GUSTAFSON (1959) and SCOTT and NYLEN, (1960). These authors suggested that the TOMES' processes of the ameloblasts were "budded off" from the rest of the cell at successive, regular intervals, and that these segments of ameloblast filled in with "enamel" to become the "boxes" (A.G. GUSTAFSON - i.e. the interval between two cross striations).

I was particularly intrigued to see if the so-called enamel tubules in marsupial enamel represented a minor or a major diversion from a general pattern of enamel development, and to find what this diversion was.

The species which have been studied are listed in Table 1. The conventional histological material (whole or half- heads, or tooth germs in situ in the jaws)was formalin fixed; cellcidin or paraffin wax-embedded; sectioned at 8-15μ& stained with haematoxylin and eosin, or 24 hour haematoxylin and eosin, or a "Trichrome" technique.

The technical procedures involved in obtaining and preparing the material studied by electron microscopical techniques (and all techniques other than "routine", light-microscope histology) have been performed by myself. All the material under this heading has been derived from tooth germs dissected from living, or recently dead animals. In the case of the larger mammals studied it was not possible to fix the tooth-germs soon enough after death to obtain satisfactory preservation of the ameloblasts. However, it was considered quite satisfactory material for the study of the developing enamel itself. The only details which can be seen within the young enamel itself, are, after all, the size and orientation of the crystallites (the organic matrix remaining conspicuously invisible - SCOTT and NYLEN, 1962) and these factors are unlikely to be affected by post-mortem changes to any extent significant to the purposes of this study.

(CHAPTER 2, SECTION 2)

MATERIALS. List of material examined in the course of the study of the development of mammalian enamels.

Mammalia		No. of animals	No. of tooth germs	Source of animal or already prepared material	E/M Cytol.- ++ good + satis. crystals only	Fixation: p = Palade's d = Dalton's k = $KMnO_4$ f = Formalin	L/M Cytol. ++ good ++ satisfactory	No. of wax reconstructions	S.E/M surface ++good +satisfactory	No. of E/M blocks
PRIMATES	Homo sapiens	12	24	CH	+	pdk		1		70
	Rhesus macacus	3	20	LH	++	pdk	++	2	++	40
PROBOSCIDEA	Loxodonta africanus	1	1	LH	-	f				24
SIRENIA	Trichecus latirostris	3	6	Z(2) PR(1)	- -	fpk		1	++	48
UNGULATA	Sus domesticus	2	10	RVC CB	- ++	pdf	++	1		70
	Bos bovis	1	1	JB	-	alc.				20
	Capra hircus	1				f	++			
	Equus caballus	1	1	RVC	-	f				36
CARNIVORA	Felis domesticus	1	14	CB	++	pd	++	1		14
RODENTIA	Rattus norvegicus	5	20	LH	++	pd.	++	3		20
	Myocastor coypus	1	4	LH	++	p	++	2	+	34
LAGOMORPHA	Lepus cuniculus	4	14	LH	++	p				14
INSECTIVORA	Erinacaeus europaeus	1	5	RA	++	pd				5
MARSUPIALIA	Didelphis nudicaudata	4	68	Z(2) LH(2)	- ++	f pd	++	2		70
	Didelphis virginiana	1		CI			++			
	Trichosurus vulpecula	1 1	8	Adel TC	+	p	++ +			24
	Wallabia rufogrisea	1		LH			++			
	Pseudocheirus convolutor	5		LH			++			
	Macropus (? species)	1		LH			+			
Reptilia	Caiman sclerops	1	8	LH	+	p	++			8

Sources of this material.

CH.....Mr. Cradock Henry, The Great Ormond St. Hospital for Sick Children.
LH.....Local: The London Hospital Medical College Research Funds or reserve material stored in the Anatomy Dept.
Z.....The Zoological Society of London, Prosectorium or Pathology Depts.
PR.....Dr. Clive I. Mohammed, The Dental School, San Juan, Puerto Rico.
RVC.....The Royal Veterinary College, London.
CB.....Mr. Carl Boyde M.R.C.V.S.
RA.....Mr. R.C. Adams.
JB.....Mr. Joseph Backhouse.
TC.....Thornton Carter's material.
CI...Dr. Burns; Carnegie Institute, Baltimore, Md., U.S.A.
Adel.Zoology Dept., Adelaide, Australia.

2.3 METHODS

2.3.1. Light microscopy - Decalcified Sections. Most of the material under this heading which I have examined has been borrowed, or prepared for me by the technical staff of the Departments of Anatomy and Dental Histology. The details of preparation are not central to this thesis and will be mentioned, where relevant, in connection with the results obtained.

2.3.2. Ultrathin sections for electron microscopy. FEARNHEAD (1961 A) attributed his success in preserving the cell membrane of the TOMES' process of the rat ameloblasts to his use of an Araldite embedding technique. It is widely recognised, now, that epoxy resins are more suitable materials than methacrylate for embedding soft tissues prior to ultramicrotomy because (a) there is less risk of "polymerisation damage", i.e. actual physical disruption of cytoplasmic architecture and (b) because Araldite is not so easily evaporated out of the specimen during bombardment by the electron beam. My choice of methacrylate as an embedding medium throughout this investigation, therefore, requires some justification - this can be done on two counts:-

First - It is "normal" procedure is fixing living material for "electron-microscopy" to cut the tissue into very small (say 500μ^3) pieces prior to its immersion in the fixative. However, the attachment of the ameloblasts to the surface of the developing enamel is notoriously easy to disrupt (FLEMING, 1958, 1961). The thickness of the enamel organ, in say, a rat incisor, is of the order of 200-300μ; that is, if the whole tooth germ is dissected out and fixed, - assuming a plane of cleavage at or near the external enamel epithelium - the fixative has no further to penetrate to the inner ends of the ameloblasts, than to the centre of a "conventional block" of tissue. To avoid any more disruption of the ameloblast layer from the surface of the enamel (than that occasioned by the process of dissecting out the tooth germ itself), the developing teeth were not comminuted into smaller blocks, except in the case of very large teeth which would not otherwise have been accommodated in the gelatin capsules used as the mould for embedding in the routine of our laboratory.

4 Second -Having decided to use very large "blocks" of tissue (by any electron microscopical standard) it did not take long to discover that, whereas a satisfactory permeation by, and hardening of,methacrylates could be achieved, it was impossible to use Araldite because the centre of the blocks never hardened. This is due to the high viscosity of the Araldite embedding mixture, which did not replace the solvents (in large pieces of enamel) which were used to displace water, before it polymerised.

2.3.2.2. Fixation. The fixative used were DALTON'S (1953) and PALADE'S (1952). In the earlier stages of this investigation both of these two fixatives were used - each to fix half the material obtained on any given occasion. Later, PALADE'S fixative was used exclusively.

The tooth germs were immersed in fixative either immediately, or after a brief rinse in buffer, and "fixed" for a period of from one-half an hour to two hours. They were then rinsed with three changes of buffer (5 mins. each) and then dehydrated in a methyl alcohol series - 30%, 50%, 60%, 80%, 90%, 95% and three changes of absolute alcohol (15-30 mins. each stage).

2.3.2.3. Embedding. The tissue was transferred from absolute methyl alcohol to a 50/50 mixture with methacrylate monomer. After a little empirical investigation it was found that a mixture of 2 parts of butyl methacrylate to 1 part of methyl methacrylate monomer gave a block hardness suitable for the sectioning of "soft" enamel. Pure polymethyl methacrylate was undoubtedly too hard, and the mixture in routine use in the department viz., 4/1 butyl/methyl/ methacrylate, rather too soft.

A few blocks were embedded in a water soluble glycol-methacrylate medium, which, however, proved to be extremely brittle ; it was not possible to obtain satisfactory sections from this material.

2.3.2.4. Ultramicrotomy. Knives. At the beginning of this investigation FEARNHEAD (1960, D) was still very keen upon the idea of preparing sections of fully mineralised enamel via the medium of a diamond knife edge. However suitable these knives may have been for such really hard material, I achieved greater success with glass knives on developing enamel. The investigation was restricted to tissue which was

soft enough to be cut with glass knives.

Ultramicrotomes. The first model to be used was a very early PORTER-BLUM type in which the block advance was occasioned by the thermal expansion of an invar rod and the "by-pass" was to one side. The thermal expansion was controlled by the nearness of the desk lamp used to heat the invar rod, and, of course, the rate of production of sections. I modified this machine by replacing the metal by-pass guide with one made of Poly-Tetra-Flour-Ethylene - this was satisfactory in operation.

For four months I borrowed an L.K.B. Ultratom (by courtesy of Messrs. Gallenkamp's helpful agent, Mr. D.E.L. Price). This ultramicrotome works on the HAANSTRA principle - magnetostriction of a nickel yoke being used to retract the block on the return stroke- the thermal expansion caused by the current flowing through the coil surrounding the nickel yoke being sufficient to provide the block advance on the cutting stroke.

At the end of this period I returned to working with a HAANSTRA type microtome built in The London Hospital Research Workshops (for R.W. FEARNHEAD). Since the arrival of modern, mechanical advance, hand-operated, lateral by-pass PORTER-BLUM microtomes in the department I have used these exclusively.

My experiences with the different types of microtome may be of benefit to someone else who may care to work in this field (- hence their mention). The magnetostriction retraction by-pass microtomes were exceedingly irritating to operate because the block would become wetted (by the fluid of the meniscus in the "boat" on the knife) on the by-pass (up) stroke. This only occurred when cutting sections of enamel and I judge that it was caused by "whiskers" of block material for example, the enamel crystallites themselves, having been pulled out of the block face. These whiskers touch the fluid in the boat on the return stroke, because the clearance in the HAANSTRA type is only some 20-50μ. With the PORTER-BLUM type of by-pass, however, the presence of such whiskers on the block face makes no difference.

Section Thickness and Mounting. Sections containing developing enamel have been cut at a nominal thickness of 500 Å as a routine with a glass-knife in the PORTER-BLUM ultramicrotome. The "thinness" which it is possible to cut is probably determined by the average crystallite

2.6 diameter, and this in turn depends entirely on the position of the block-face in the enamel in relation to its "maturity"(Chaps.3,4). The sections were picked up on formvar coated copper grids (2.3 mm. diameter). In the latter stages of the investigation - after I had had built a Carbon Evaporation Unit - it became possible to use Formvar Films which had been strengthened with a layer of evaporated carbon, and to "sandwich" (WATSON, 1960) the sections between the support film and a layer of evaporated carbon. This was an undoubted improvement as there was less fear of "drift" during exposures, and less loss of the embedding medium from the sections.

2.3.2.5. Electron Microscopy. - The grids were examined in a Siemens Elmiskop 1 electron microscope (E/M) in the Anatomy Department (this microscope is on loan from the Wellcome Trust). The findings which are to be reported in this thesis were obtained from exposures made at the relatively low magnifications, viz:- 1,000-20,000 X initial E/M magnification and a 3X photographic enlargement during printing the negative.

All the available accelerating voltages on this microscope were used (40, 60, 80 and 100 KV). The great majority of exposures were made at 60 KV, with a condensor aperture of 100μ, and an objective aperture of 30 or 50μ. The image brightness (condensor lens setting) was adjusted so that exposures of the order of 5 seconds were usually used. Development of the exposed Ilford N50 Half Tone Orthochromatic Plates was carried out for 3 minutes in Kodak D163 developer.

2.3.3. Wax Reconstructions. Serial $\frac{1}{2}\mu$ sections cut on the PORTER-BLUM ultramicrotome were collected on carefully shaped fragments of glass cover-slips. Ribbons of (from 10-40) sections were collected on each piece of glass, and allowed to dry down - usually aided in a 40°C oven. The methacrylate embedding medium was removed in acetone (15 mins.) and the sections were stained in saturated aqueous crystal violet (15 mins.), rinsed rapidly in distilled water, stained for a further 15 mins. in concentrated aqueous basic fuchsin, rinsed in distilled water, allowed to dry, and mounted in D.P.X. A light microscope was lashed up so that drawings in projection could be made

2.7 at an initial magnification of about. 500 X. Serial drawings were made of the serial $\frac{1}{2}\mu$ sections, showing only the outline of the developing surface of the enamel. The intense basophilia of the developing "enamel" ensured its clean-cut differentiation from the cytoplasm of the ameloblast and the TOMES' process. The outline of the developing surface was cut in wax sheets - laying them over the serial drawings. The manufacture of wax-reconstructions was the only way of extracting the information contained in the serial drawings. Attempts at graphic reconstruction met with a conspicuous lack of success.

Wax reconstructions were prepared from human, monkey, manatee, coypu and opossum material. The blocks from which the sections were cut were just the routine E/M blocks and on occasion the series of $\frac{1}{2}\mu$ sections was interrupted to cut ultra-thin (500 Å) sections from the same block face. It was thus possible to relate the morphology, e.g. the crystallite orientation, as seen in the E/M, to the shape of the wax reconstructions.

2.3.4. Scanning (Reflection) Electron Microscopy (BOYDE and STEWART, 1963).

2.3.4.1. A few introductory remarks about this method of microscopy are probably in order as it is certain that this is the first application of a scanning electron microscope (S. E/M) to the study of developing dental tissues:- A finely focussed beam of electrons is made to scan a raster across an area on the specimen surface in synchronism with the spot of a cathode ray display tube. The brightness of the cathode ray tube spot (i.e. the "television" screen on which the image is seen) is modulated by a signal derived by amplification of the electron current coming from the specimen surface. Depending on the bias applied to the electron-collector, the electrons used to form the image may either be high energy "reflected" electrons with energies approaching that of the scanning electron beam, or low energy secondary electrons. (The x-rays emitted can also be used (indirectly again) to modulate the brightness of the cathode ray tube spot - see Chapter 3.) Contrast in the image is partly determined by simple topographical variations - e.g., differently sloping parts of the surface will "reflect" a greater or lesser number of electrons towards the electron

collection system - and partly by variations in composition in the surface, since electron "reflection" depends on the number of orbital electrons in the atom ("atomic number contrast"). The surface of the specimen must be conducting; otherwise it will charge up and deflect the electron beam.

Both high energy reflected electrons and secondary electrons were used to form images in the Cambridge Instrument Company "Stereoscan" developmental scanning electron microscope. The accelerating voltages employed were in the range 12-16 kV. The image was photographed on a special display screen (during a single scan of approximately one minute duration) using a Polaroid-Land Camera with a high speed paper. The extra expense of this method of recording the image is undoubtedly compensated for by the knowledge - acquired only 10 seconds after the exposure - that the exposure is satisfactory and need not be repeated. The magnification of the image is the ratio of the size of the scan on the specimen surface to the size of the scan on the cathode ray tube, and can be varied at will.

2.3.4.2. Specimen Preparation. The specimens were portions of developing teeth. The surface of the developing enamel was stripped free of ameloblasts by gently lifting off this layer with tweezers - aided on some occasions by a gentle stream of tap water. The teeth were allowed to dry, - usually in vacuum - and then stuck to the head of $\frac{1}{8}$" counter-sunk aluminium rivets with Araldite adhesive, leaving the surface of the developing enamel exposed. Gold-Palladium films were evaporated on to the specimens whilst they were tilted at 45° to the evaporation source and were rotating rapidly. This procedure ensured the removal of non-conducting "shadows" from the specimen surface. The commercially available apparatus which would have enabled this complicated process (of rotating the specimen whilst evaporating the metal) to be performed is extremely expensive because it employs a rotary vacuum seal. A very cheap electron motor fitted with a device to hold six specimen holders (Al rivets) was fitted inside the vacuum chamber so that the disc holding the specimens faced at 45° to the evaporation filament. The possible objection of the use of the

Figure 2.1. Electron micrograph (X 16900) of ultra-thin section of the junction of the outer end of the ameloblasts and the stratum intermedium cells in a tooth germ of a pouch young rat-tailed opossum, Didelphis nudicaudata at the stage immediately preceding enamel formation by these particular cells.

The outer terminal bar apparatus (terminal bars and terminal web) of the ameloblasts runs obliquely across the centre of the image. The stratum intermedium cells are attached to each other and to the ameloblasts by desmosomes.

This section was mounted on a Formvar support film and then "sandwiched" between this and another formvar film. The folds in this second film are unfortunately all too obvious.

LIST OF ABBREVIATIONS USED IN LABELLING THE ELECTRON MICROGRAPHS ILLUSTRATING CHAPTER II.

A Ameloblast
E Enamel
M Mitochondrion
N Nucleus
G Golgi zone
P Prism or Rod
D Desmosome
TB terminal bar
TW terminal web
ICS intercellular substance
TP TOMES' process
IRS inter-row sheet
IPR interprismatic region
WPR winged process region of Pattern 3 prisms
g group of crystallites with a-axes parallel
pb prism boundary plane (prism sheath)

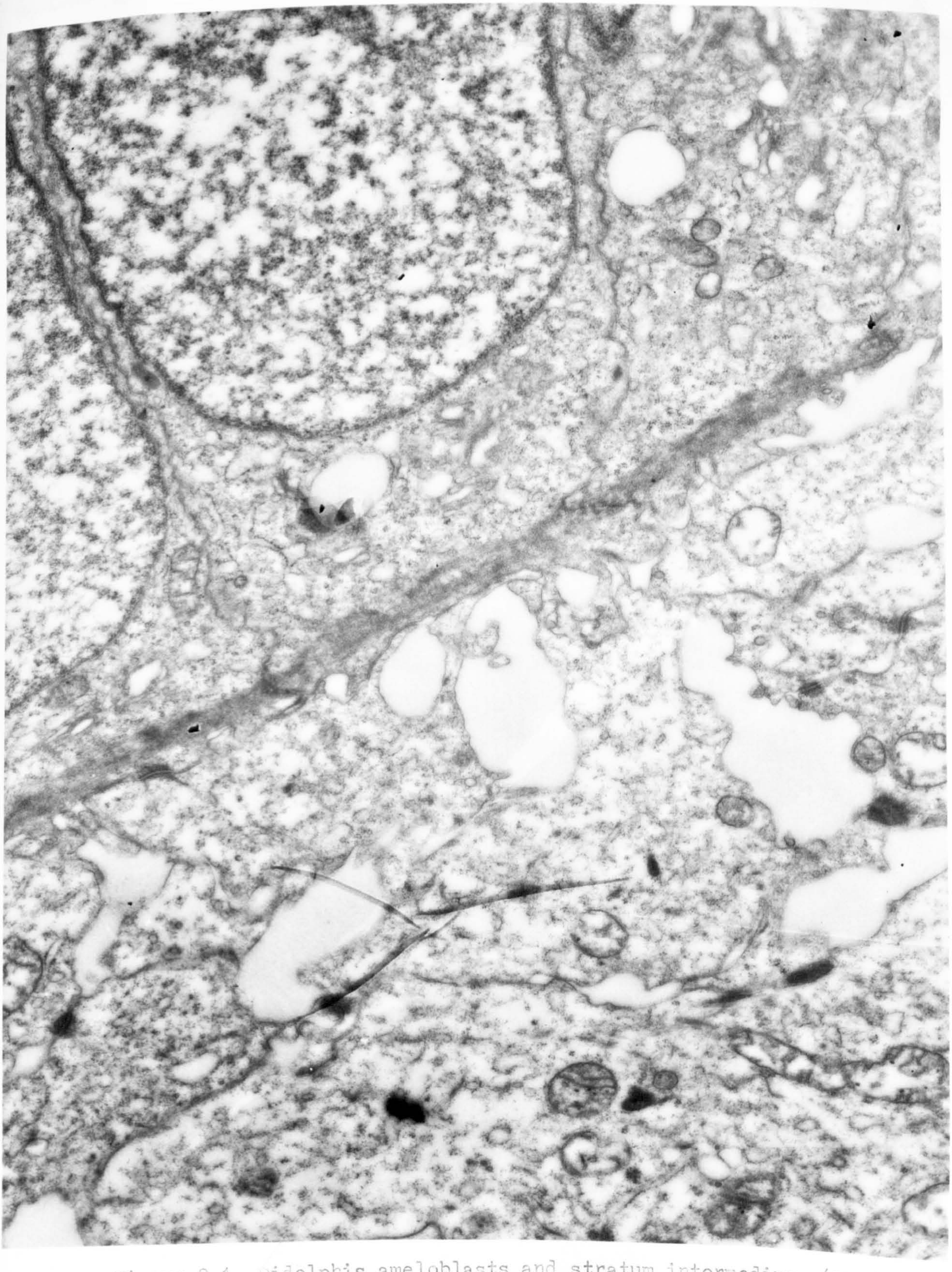


Fig 2.1

Figure 2.1 Didelphis ameloblasts and stratum intermedium. (X 16900)

2.9

the whole electric motor inside the vacuum, was, the large, expected out-gassing of the windings of the motor. However, the motor stood up to a prolonged degreasing treatment by running it in a bath of chloroform - after drying, its bearings were lubricated with diffusion pump oil and it ran perfectly well in the vacuum chamber. Evaporation of the Gold-Palladium was carried out at vacua of better than 5×10^{-5} m.m. Hg. (A.E.I. Metrovac Ionisation gauge) with the motor running and the filament switched on. After "metallising", the specimens were stored in gelatine capsules until examined in the "Stereoscan" S. E/M.

2.3.4.2. The honeycomb of the developing enamel surface can be seen clearly in the scanning electron micrographs (Fig. 2.14). A short report of these findings has already been published (BOYDE and STEWART, 1963).

2.4. RESULTS

2.4.1. Ameloblast Cytology

(1) The cell membranes of adjacent ameloblasts have been found to closely parallel each other even when corrugated. For the most part the lateral cell margins were found to be remarkable straight. A hexagonal packing of the ameloblasts is most commonly found. Intercellular spaces - not always present - are more often confined to the corners of the hexagons - that is,at the line of junction of three adjacent cells. It is very difficult to identify spaces as intercellular in other than transverse sections of the ameloblasts. The only thickenings of the cell membrane that were found, were matched by those of the adjacent cell membrane in typical desmosomes and terminal bars. (Figures 2.1; 2.2 - YAMADA, 1955; RHODIN and DALHAMN, 1956; FAWCETT, 1958). Desmosomes occur between the cell membranes of the outer ends of the ameloblasts and the adjacent (inner) cells of the stratum intermedium. The outer terminal bars and the terminal web in the cytoplasm are very close to this end of the cell (- it is hardly surprising that WILLIAMS (1896) named these structures the "outer ameloblastic membrane"). The inner terminal bars and terminal web define the "junction" between the ameloblast body and the TOMES' process, and so are situated some several (5-15) microns from the end

Fig. 2.2. Electron micrograph (X 16000) of a near-tangential section of the surface of developing cat (Felis domesticus) enamel; near transverse section of the inner ends of the ameloblasts. Note the "honeycomb" of "enamel", the terminal bar apparatus, the prominent mitochondrion in close juxtaposition to the terminal bar apparatus, and the intercellular spaces further out (to the right).

The streaks running across the image from WNW to ESE are caused by variations in the thickness of one of the sandwiching, supporting Formvar films: there are also some small folds in one of these films.

Fig. 2.3. Electron micrograph (X 15500) of a transverse section of rat incisor ameloblasts during inner-enamel formation, showing the proportion of the cell diameter occupied by the nuclei. The nuclei tend to reflect the cross-sectional shape of their respective ameloblasts. A prominent nucleolus can be seen in the nucleus at the centre of the left hand side of the field.

Fig. 2.4. Electron micrograph of longitudinal section of Pig ameloblasts showing the typical outline of the nuclei in this plane of section.

The field extends from the ameloblast nuclei at the left, to two capillaries surrounded by stratum intermedium at the lower right. Note the density of (poorly fixed) mitochondria in the supra-nuclear pole of the ameloblasts and the numerous mitochondria, microvilli and intercellular spaces in the stratum intermedium.

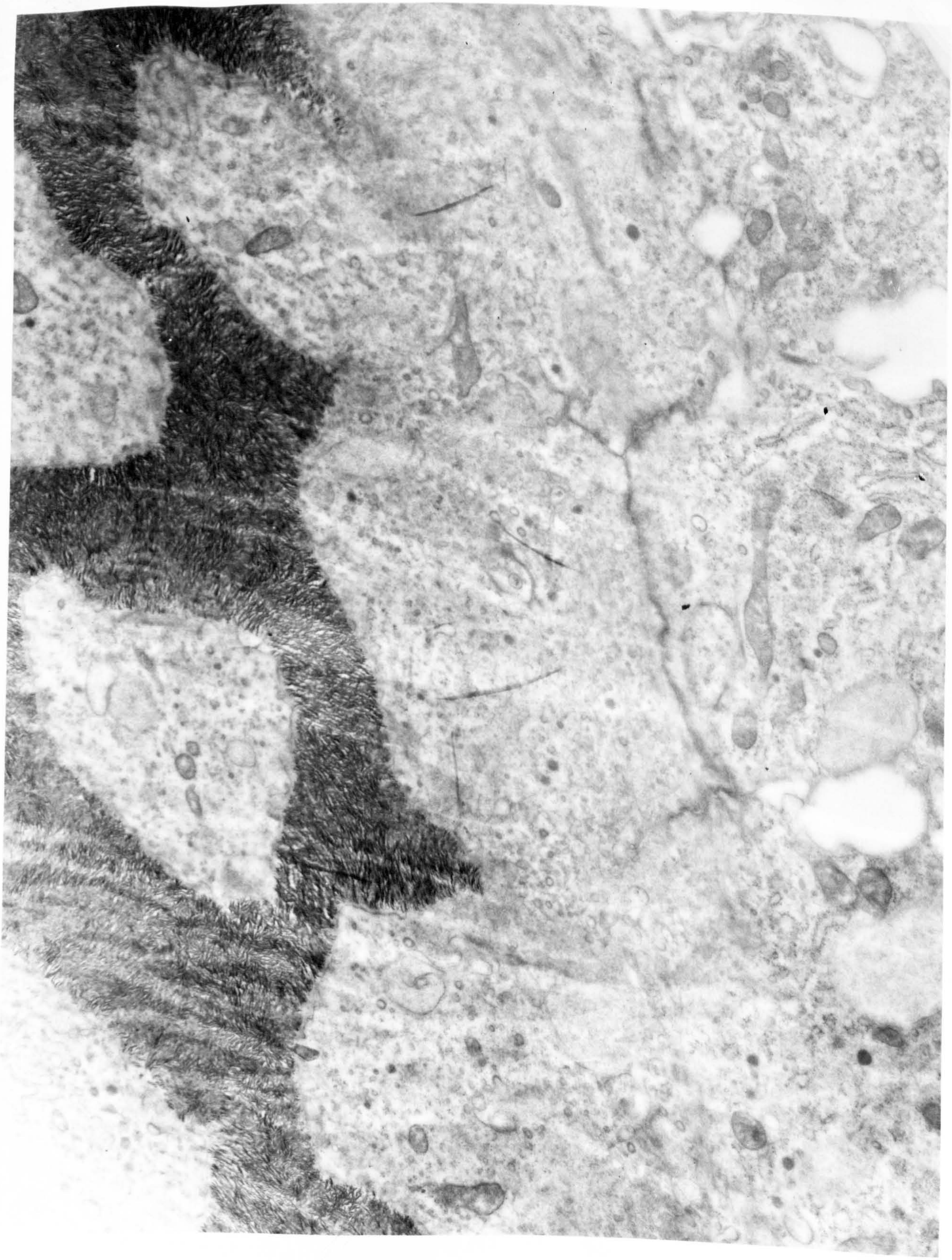


Figure 2.2 Felis: mineralising front and ameloblasts. (X 16000)

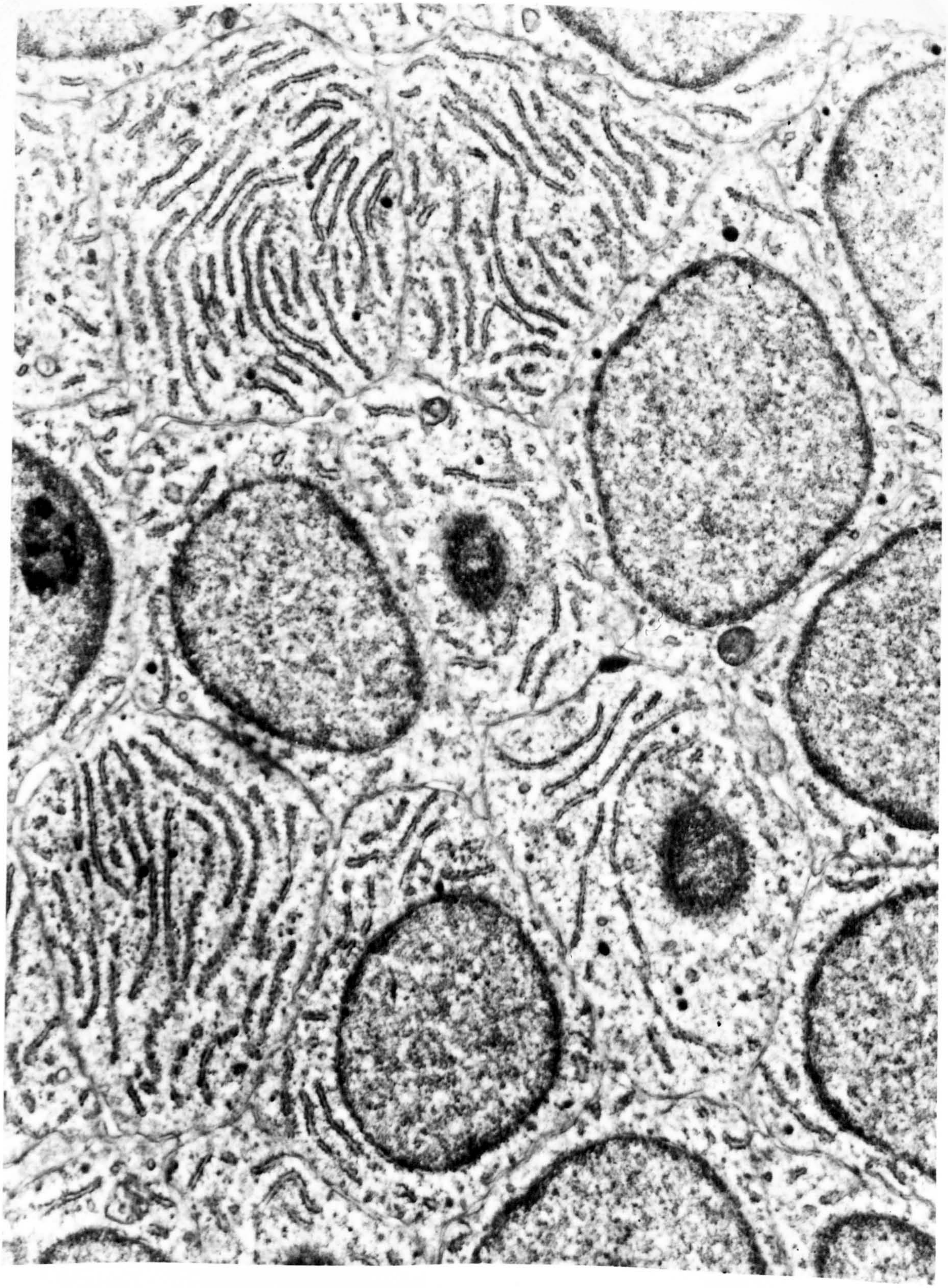


Fig 2.3

Figure 2.3 Rattus: T.S. ameloblasts (nuclei) (X 15500)

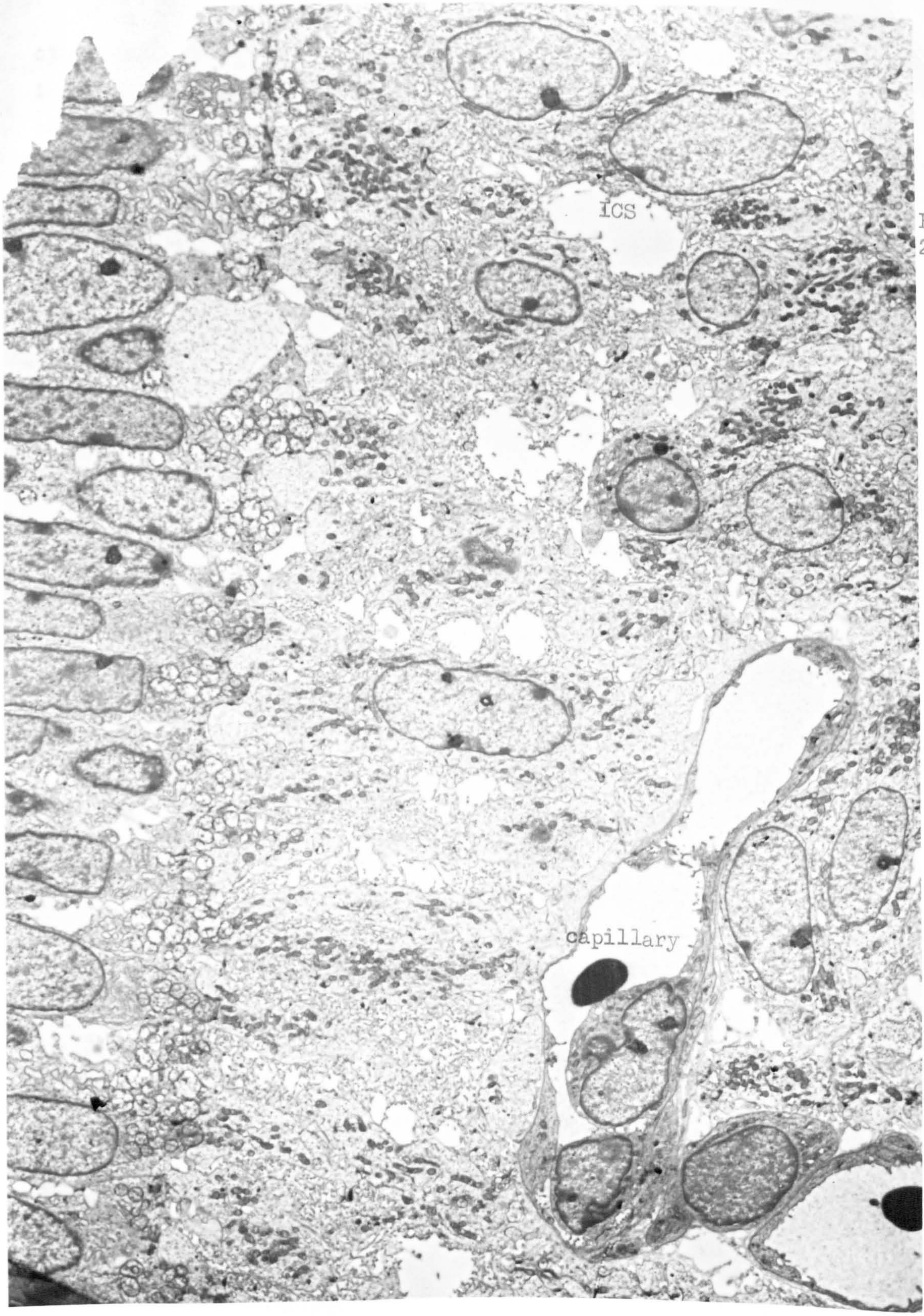


Fig. 2.4.

Figure 2.4. Sus: ameloblasts and stratum inter ium. (X 3360).

of the cell itself. Occasionally, short microvilli project into the intercellular spaces between ameloblasts, and between the ameloblasts and the stratum intermedium cells (as also into the intercellular spaces between stratum intermedium and stellate reticulum cells).

2.4.1.2. Nucleus - The nucleus has a double membrane - with pores - some fairly dense aggregations of the granular material of the nuclear matrix are attached to the inner layer. Aggregations of granules or single granules (ca.70 Å) are attached to the outer layer of the nuclear membrane, so that the membrane is most clearly defined by the electron transparent divide between two halves of the membrane. A nucleolus can usually be found, and it is often in close relationship to the aggregations of granular material on the internal layer of the nuclear membrane. The nucleolus can be recognised by its greater opacity, apparently due to the closer aggregation of its constituent particles. There are often very dense aggregations within the nucleolus.

The nuclei are almost round in cross-section and any deviations from this are generally faithful reproductions of variations from the normal,hexagonal, cross-sectional shape of the cell itself, since the nucleus occupies the greater part of the cell diameter at its own level. As a typical example one might take Figure 2.3 in which the cells are 6μ in diameter and the nuclei 5μ - thus leaving an approximately $\frac{1}{2}\mu$ wide seam of cytoplasm all round the nucleus. In longitudinal section (Fig. 2.4) the nuclei are appreciated as ovoid bodies with rather flattened ends. A crenation of the nuclear outline and a more concentrated clumping of the nuclear granular material have been found in ameloblasts nearing the completion of active enamel secretion, and is always found in the "maturing" ameloblast.

2.4.1.3. Golgi apparatus and secretory granules. A prominent Golgi zone consisting of an extensive aggregation of smooth-surfaced, flattened membranous sacs and vesicles, is found in a position just below the nucleus in the secreting ameloblast. The Golgi zone is particularly prominent in the rat incisor ameloblast (KALLENBACH, et al 1963) and a semilunar configuration of its elongated, flattened sac-like

Fig. 2.5. Electron micrograph (X 15000) of transverse section at the level of the Golgi zones of rat incisor ameloblasts during inner enamel formation. The lateral cell surfaces are smooth and parallel in this case. back 2.10.

Fig. 2.6. Electron micrograph (X 19900) of longitudinal section of rhesus monkey deciduous molar ameloblasts at a level between the Golgi zones and the inner terminal bars. Note the longitudinal array of α-cytomembranes; the longitudinal disposition of the elongated mitochondria; the absence of α-cytomembranes and mitochondria from the peripheral zone of these cells (particularly clearly seen in the wide band across the centre of the field which cuts the meeting planes of a number of cells at a tangent); and the complexity of the lateral cell surfaces in this particular preparation.

Fig. 2.7. Electron micrograph (X 15300) of transverse section of pig ameloblasts (from developing edge of enamel of upper first permanent molar at a stage when the cusps were still separate). Note the transversely sectioned mitochondria and the intercellular spaces at the "corners" of the ameloblasts.

This field is part of a montage which demonstrated that the preferred orientation of the α-cytomembrane array (WSW - ENE) corresponded with the direction of the longitudinal rows of ameloblasts and (Pattern 2) prisms in the forming enamel in this case. However, the ameloblasts are not sectioned quite transversely, and the "longer transverse axes" of these cells also corresponds with the direction of orientation of the α-cytomembranes; this may be part of the explanation of the appearance of this orientation.

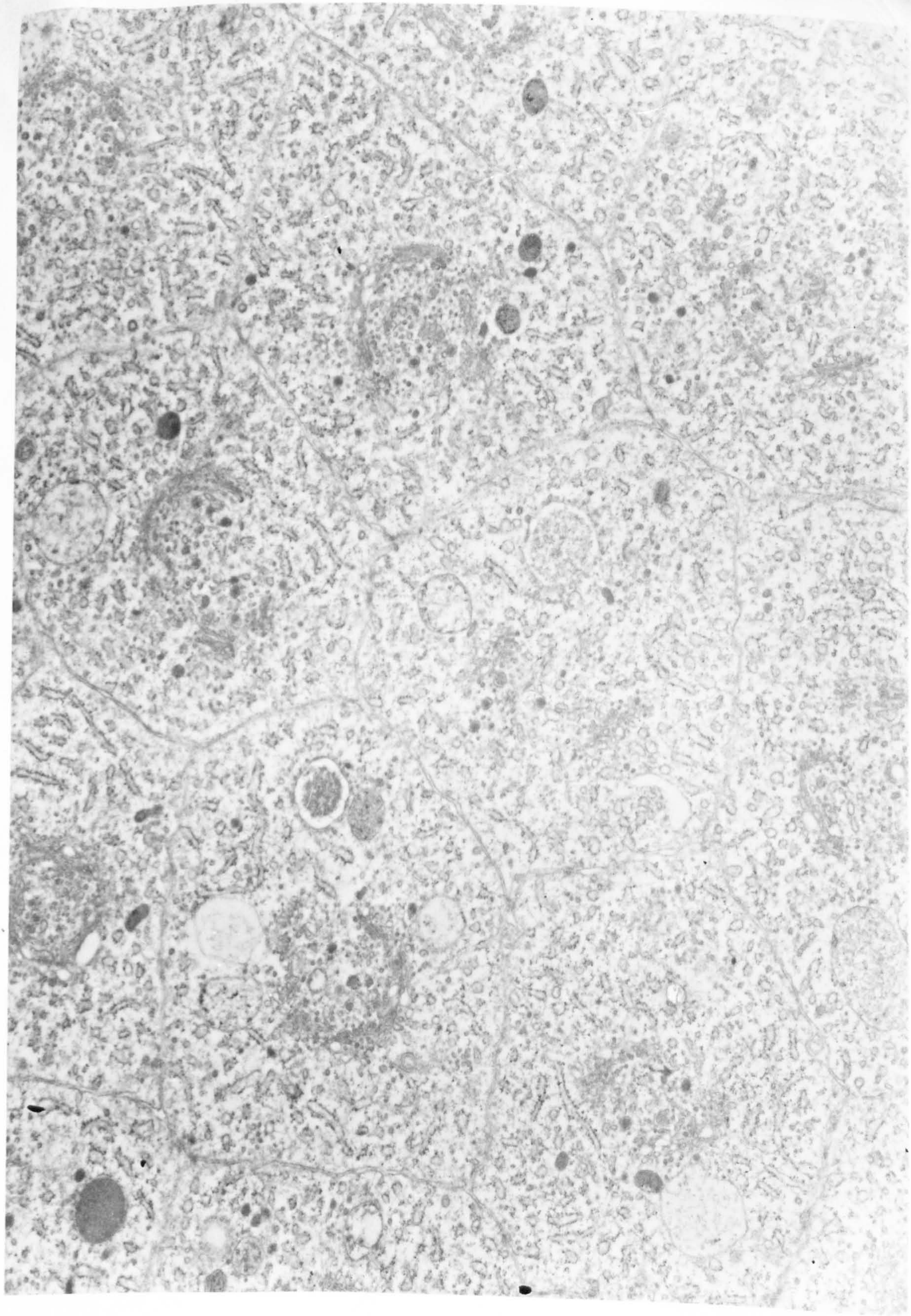


Figure 2.5 Rattus: T.S. ameloblasts (Golgi zones) (X 15000)

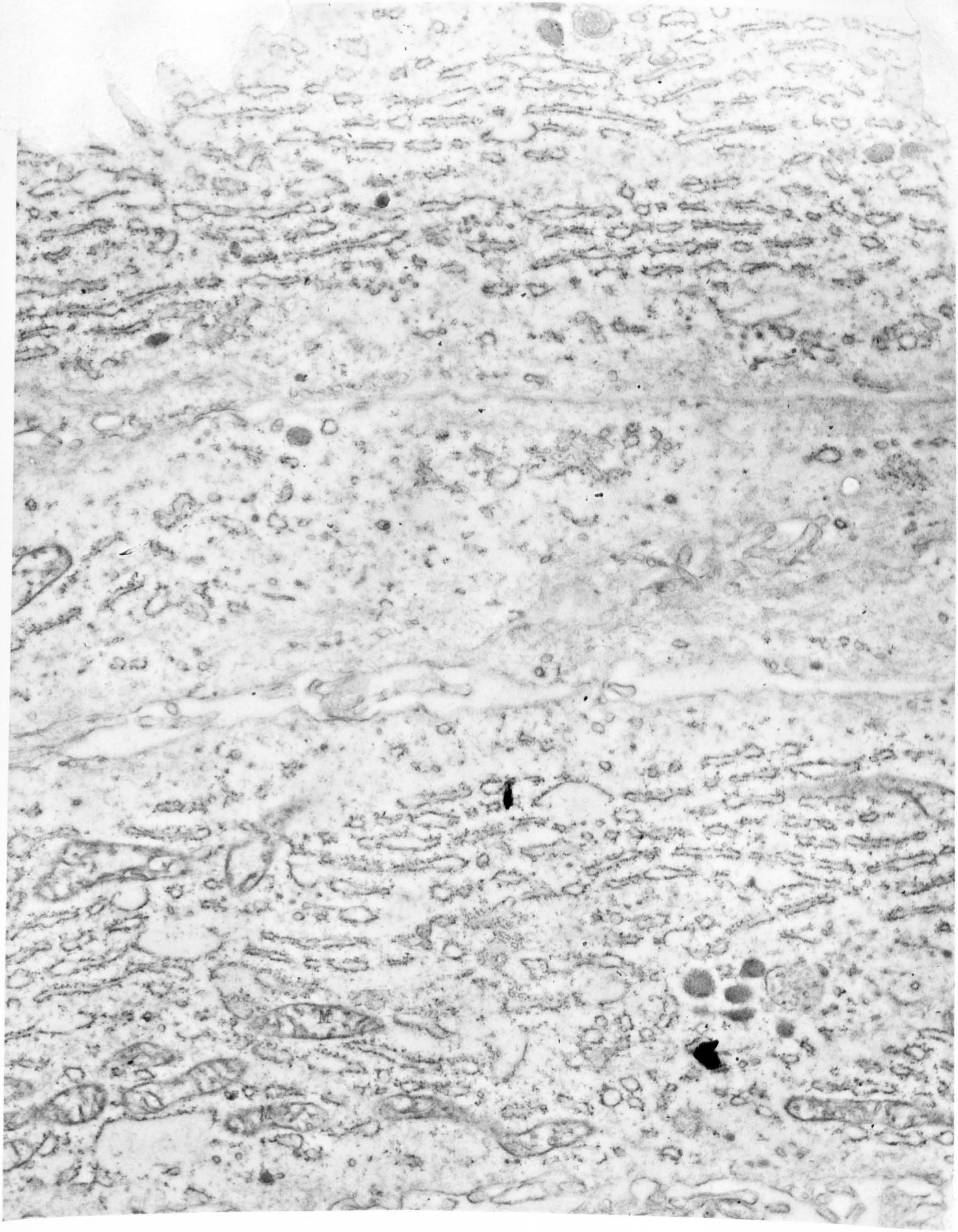


Figure 2.6 Rhesus: L.S. ameloblasts (infranuclear) (X 1990

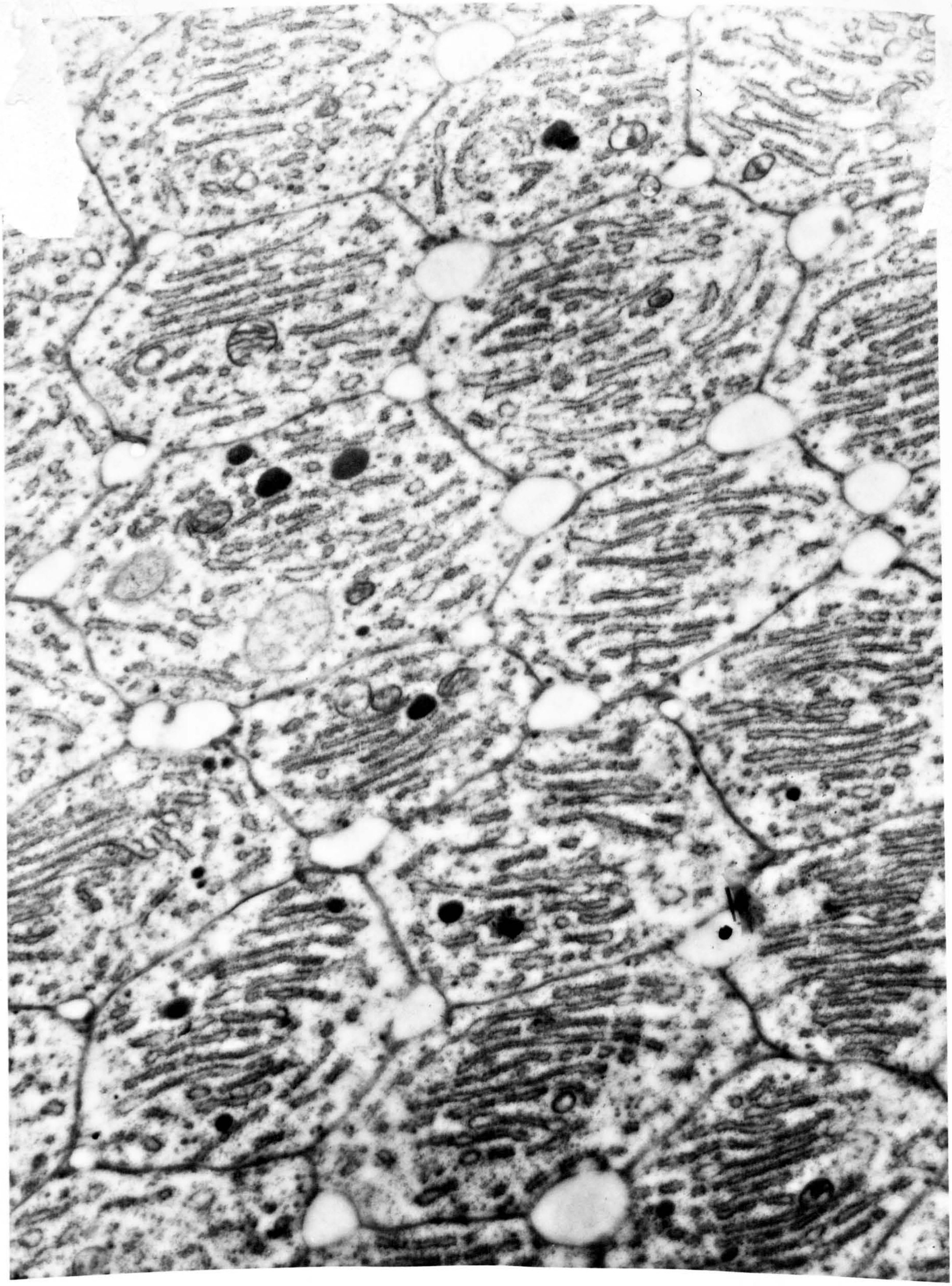


Figure 2.7 Sus: T.S. ameloblasts (infranuclear). (X 15300)

2.11

component, surrounding (it its centre) the vesicular component, can nearly always be found in transverse sections of this cell (see Fig. 2.5). The density of the contents of the finest (ca. 200-500 Å) diameter, and incidentally the most numerous, vesicles varies from quite electron-transparent, to dense. For me, the next most eye-catching components of the Golgi region are the ca. 1,500 Å diameter, membrane bound "granules" with a dense granular matrix. These granules usually have a "transparent" zone between the rim of their granular content and the bounding membrane. Next most prominent, though present in greater numbers, are the ca. 1,000 Å diameter "granules" or vesicles containing a pale, amorphous material. I have also found the much larger (ca. 1-1.5μ) diameter granules described by REITH (1960) as his Types, A, B, C and D (see Chap. 10). However, I am a bit dubious about the existence of REITH'S type B granule, in which no internal structure is discernable. All these (just mentioned) types of granule may be found in the Golgi region and throughout the cytoplasm towards, and in, the secretory pole of the cell.

2.4.1.4. <u>Endoplasmic Reticulum and "loose" RNA particles</u> The most prominent development during the differentiation of the ameloblast, is that of the rough-surfaced, flattened membraneous sacs of the endoplasmic reticulum (∝-cytomembranes), which come to pack out the whole body of the cell (though mostly below the nucleus) to the terminal web. (Figure 2.6).

The well marked longitudinal orientation of the ∝-cytomembrane array has been noted previously (e.g. SCOTT and NYLEN, 1958; Fig.2.6) but I am not aware that any comment has been made on a common pattern of its orientation in adjacent cells as appreciated in transverse sections. I have, on occasion, noted (and particularly in the rat and pig (e.g. Figs. 2.7., 2.8) ameloblasts) that there is a predominant orientation of the ∝-cytomembrane array along one transverse axis of the cell. In the rat incisor ameloblasts, this direction may alter in alternate rows of cells, at least over limited fields.

RNA granules are not confined to the surface of the ∝-cytomembranes. Large numbers of scattered clumps or "rosettes" of (typically) six of these granules are scattered throughout the cytoplasmic matrix.

Fig. 2.8. Electron micrograph (X 17000) of transverse section of rat incisor ameloblasts (during inner-enamel formation) at a level between the Golgimzones and the inner terminal bar apparatus.

back 2.11.

Fig. 2.8.B. In lower power micrographs (covering a larger field) it is sometimes possible to determine a relationship between the orientation of the α-cytomembranes and the direction of the rows of ameloblasts related to the formation of the rows of prisms in the inner-enamel. The prominent dark lines crossing this image were caused by folds in the formvar support film.

Fig. 2.9. Electron micrograph (X 20800) of a section approximately normal to the surface of rhesus monkey enamel during its maturation. Note the clumping of the ameloblast nuclear chromatin; and the large number of mitochondria in the inner ends of the ameloblasts. The cytoplasmic fixation (Palade's method) is poor.

The crystallites in the (true surface zone) enamel lie approximately perpendicular to the surface. Just outside the enamel surface (i.e. below it in this field) there is a layer of amorphous or finely granular (grey) material which may have been displaced from the enamel and/or may represent the material or zone which will be the enamel cuticle at a later stage of development. REITH (1964, personal communication) has micrographs of maturing rat incisor ameloblasts which show membrane bound granules containing the same amorphous material actually within these cells.

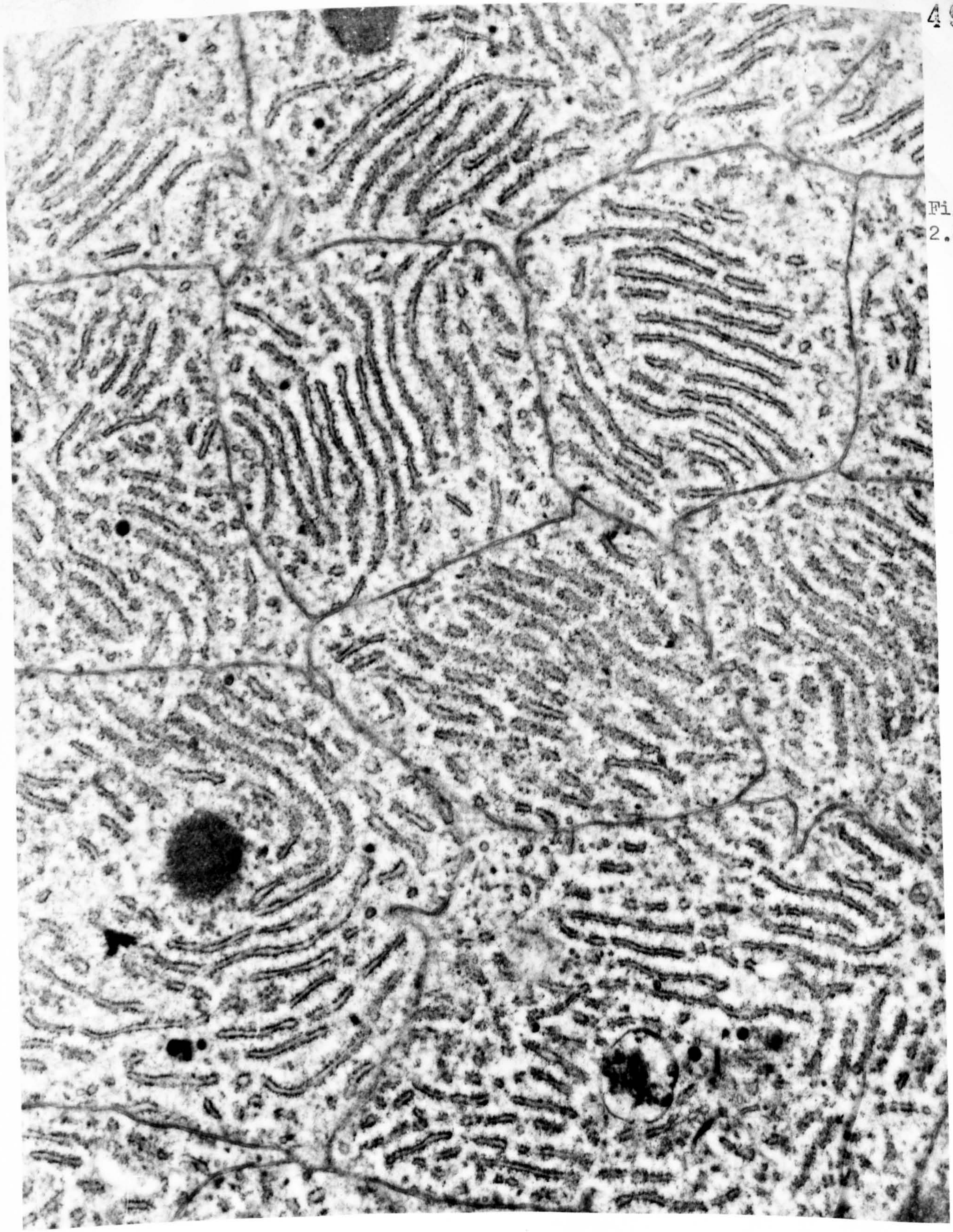


Fig 2.8

Figure 2.8 Rattus: T.S. ameloblasts (infranuclear) (X 17000)

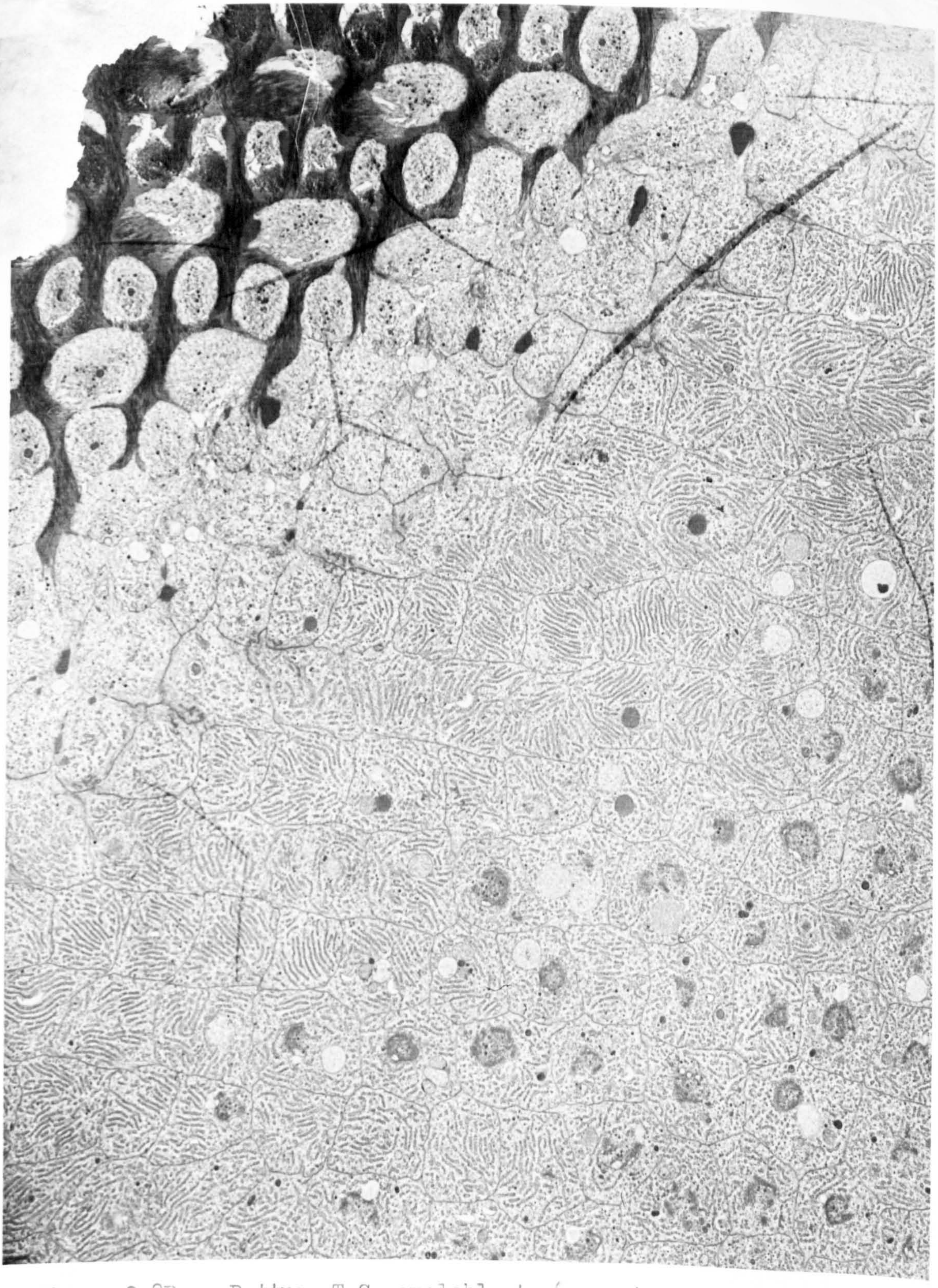


Fig. 2.8B

Figure 2.8B. Rattus: T.S. ameloblasts (near tangential section of surface of developing enamel). Note transverse (i.e. left to right) rows of ameloblasts. (X 4200)

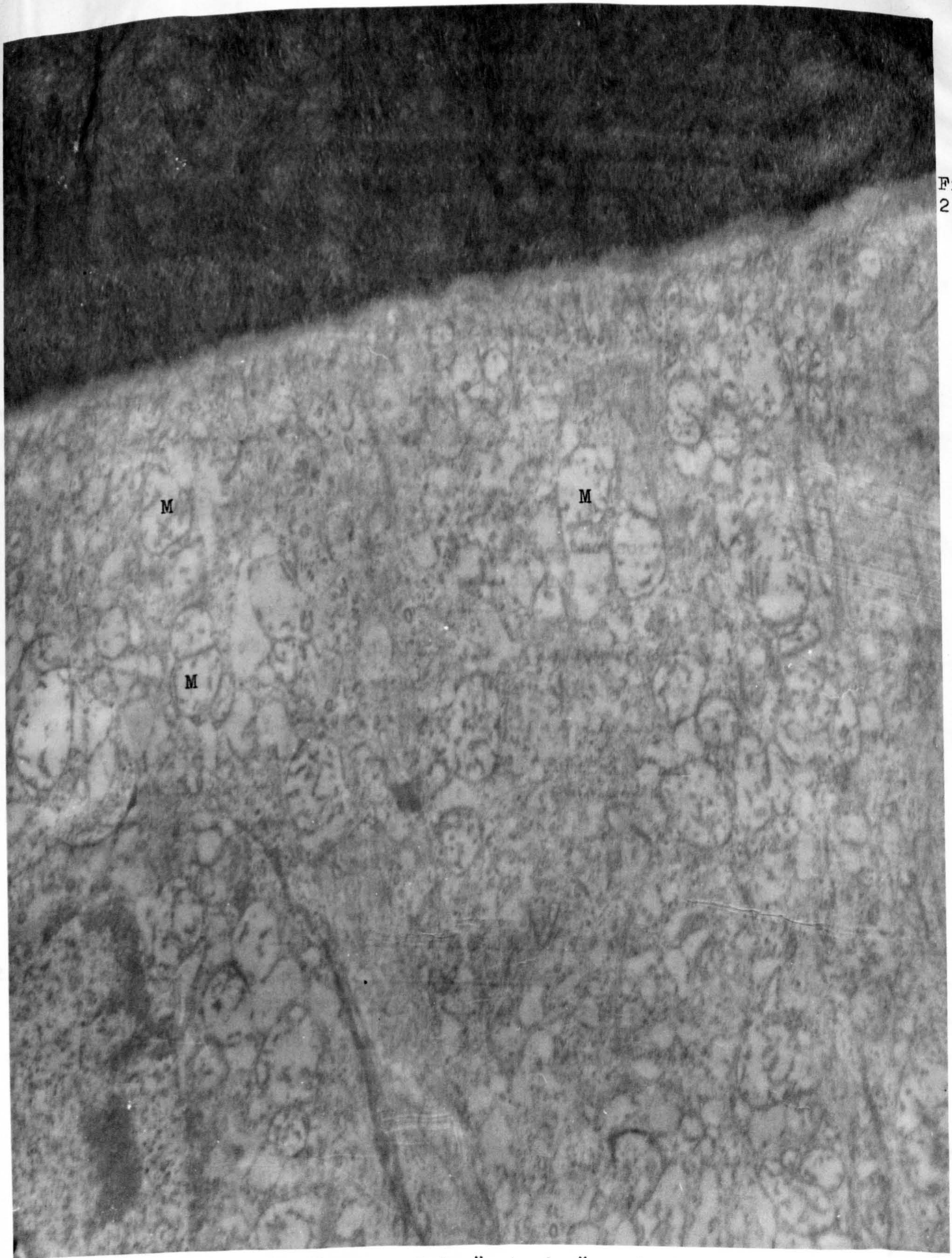


Fig. 2.9.

Figure 2.9. Rhesus: L.S. "maturing" ameloblasts.

——————— (X 20800)

.12. 2.4.15. Mitochondria - (Closed double-walled vessels with their inner walls thrown into folds). The mitochondria are elongated, sausage-shaped structures about $\frac{1}{2}\mu$ in diameter and sometimes several microns long. The mitochondria in the infra-nuclear cytoplasm are more common than descriptions of the ameloblast architecture given by previous workers would lead one to suppose: they appear to be longer than the more numerous, crowded mitochrondria in the supra-nuclear pole of the cell. This may be due to the fact that the infra-nuclear mitochondria are well orientated straight and parallel with the longitudinal array of the α-cytomembranes and that it is thus possible to section them in their length: they appear circular in cross-sections of the infra-nuclear regions of ameloblasts.

2.4.1.6. Maturation (Fig. 2.9). The changes in the ameloblast cytoplasm associated with the maturation of rat incisor enamel have been well described by REITH(1961). These changes (Fig. 2.9) consist essentially of an increase in the amount of intercellular space: the development of numerous microvilli; the endoplasmic reticulum becomes dilated and finally unrecognisable as such; and the inner ends of the ameloblasts acquire a great number of mitochondria. Maturing ameloblasts were studied in the human, hedgehog, Trichosurus, and rat - the changes outlined above were found in all these species: REITH (1961) considered that they indicated a change from a secreting to a "transporting" function for these cells.

2.4.2. (Results) The structure of developing enamel

2.4.2.1. Extracellular nature of enamel development

In all the material which was satisfactorily preserved it was possible to confirm the presence of a cell membrane between the enamel and the ameloblast cytoplasm, and further that there is a layer (of variable thickness) of extracellular "granular" material (FEARNHEAD, 1960; "stippled material" of WATSON, 1960) between the ameloblast cell membrane and that part of the enamel containing the first, of exceedingly fine diamater, "needle-like" crystals of hydroxyapatite. (Figs. 2.2, 2.16, 2.26).

Figure 2.10.

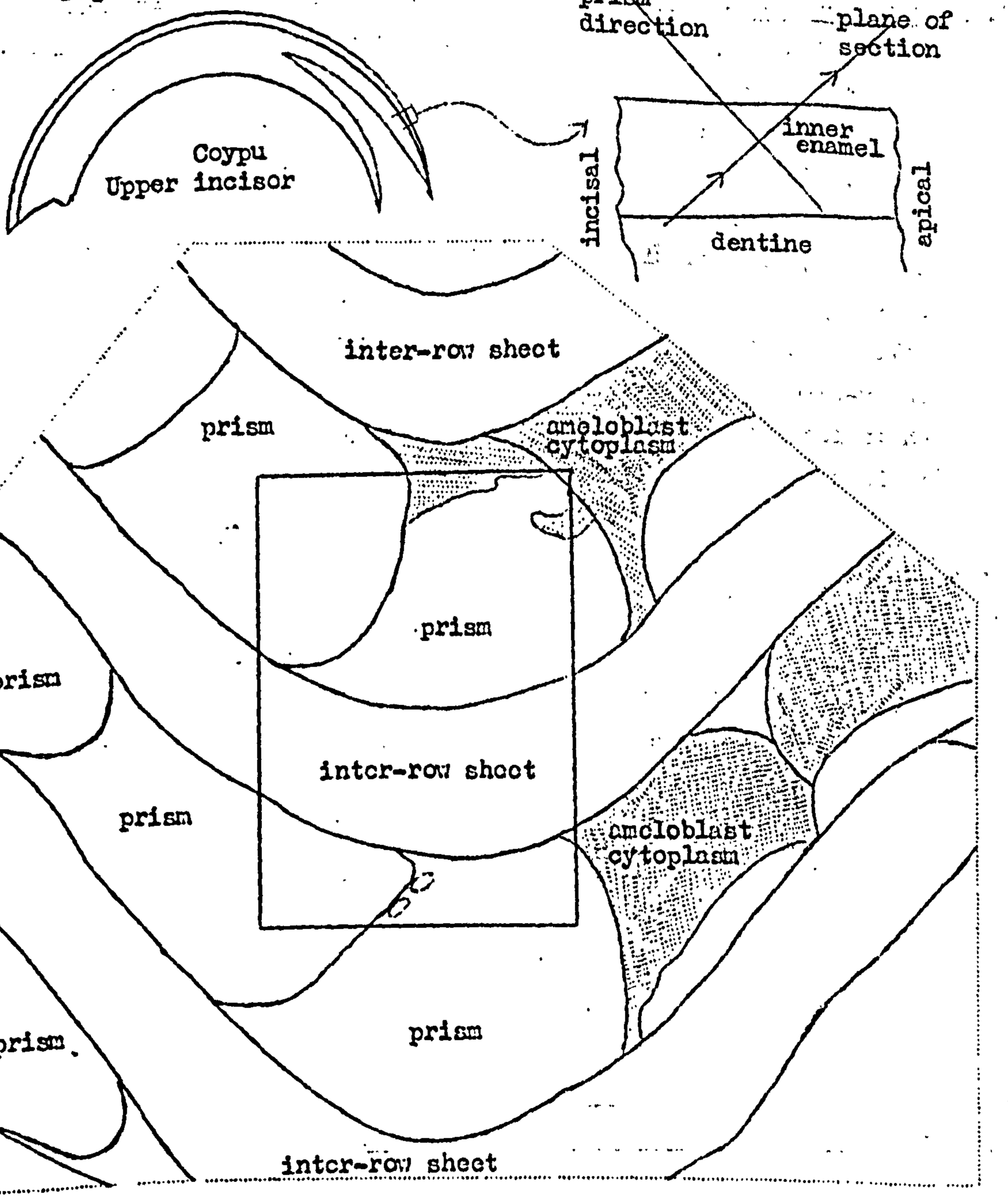


Fig. 2.10. (opposite) Electron micrograph (X 37800) of section of Myocastor coypus inner enamel taken very close to the mineralising front; showing groups of groups (g.g.) of crystallites sectioned transversely . The longer transverse axes of the crystallites are parallel in those groups.

(above centre) Tracing of same micrograph (X 15000) showing location of the enlarged field opposite.

(top of this page) Diagram illustrating approximate location and plane of section of this field. The plane of section is roughly perpendicular to the average prism direction.

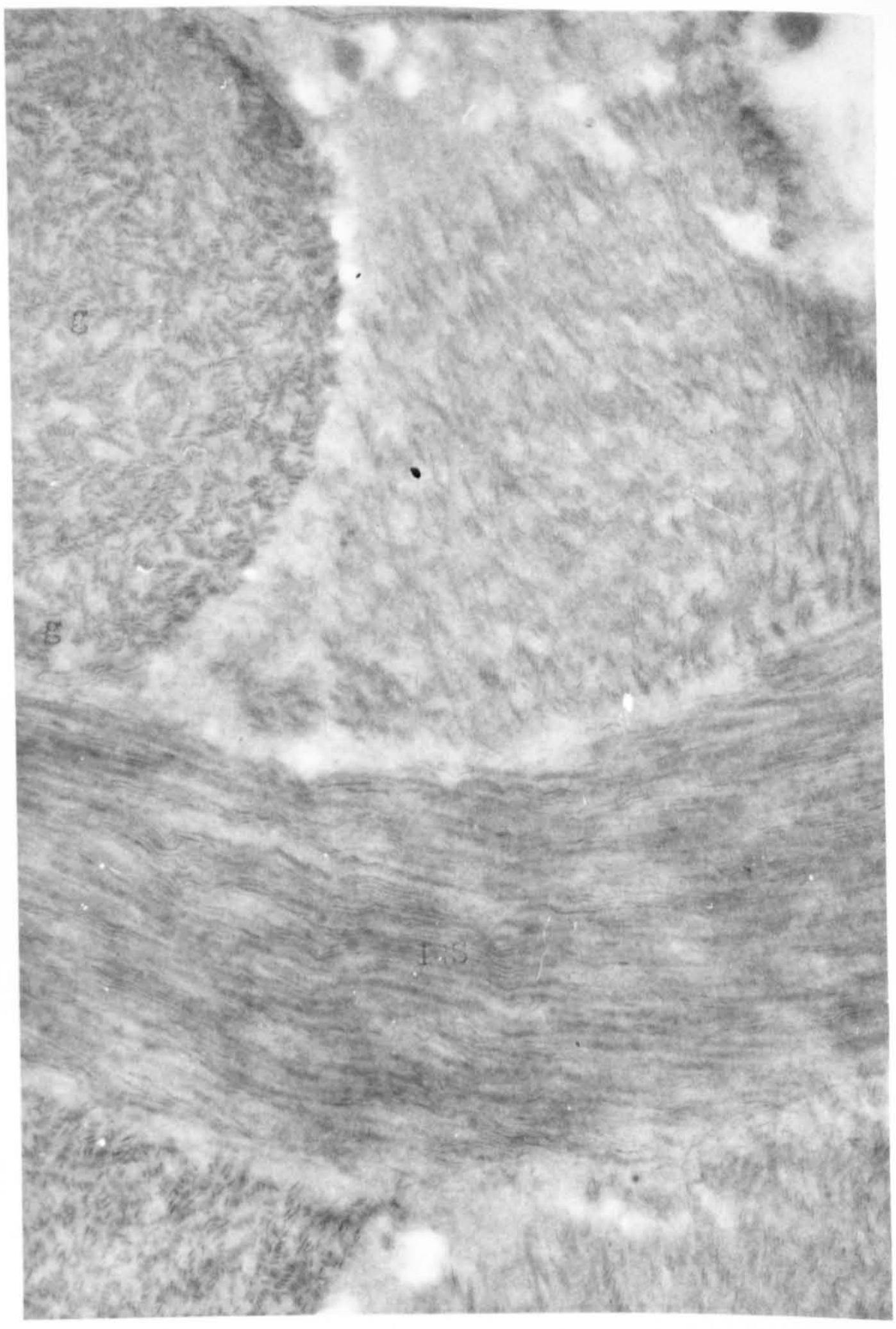


Figure 2.10. Myocastor coypus: developing enamel prisms T.S.

——————————— (x 37800)

.13

2.4.2.2. Morphology of crystallites - The change in morphology of the enamel crystallites from the first formed, exceedingly fine diameter, "needle-like" particles to the larger diameter, "tape-like" or "ribbon-shaped" crystallites - the characteristic shape found in ultra-thin sections of developing enamel that is still just "soft" enough to be cut with glass knives - has now been described many times (e.g. FEARNHEAD, 1960; RÖNNHOLM, 1962; NYLEN, EANES and OMNELL, 1963). The same sequence has been found in all the species studied here.

The tendency for the tape-like crystallites to be arranged in "groups" with their greatest-area surfaces adjacent was noted by RÖNNHOLM (1962). I have also seen this arrangement in much of the material examined. It is best appreciated in transverse "sections" of the crystallites (Fig. 2.10). This means that the crystallites are oriented parallel to one another with respect to both their c-axes and a-axes, over limited fields. No correlation between morphological features and the orientation of the crystallite a-axes has been found. In the following sections - which contain the findings relating the orientation of the c-axes to the mineralising front in the developing enamel - it will be assumed that only the orientation of the c-axis is being discussed.

2.4.2.3. The shape of the surface of developing enamel - its relationships to the ameloblasts, "prisms" and details of crystallite orientation.

I have made the distinction between prism- and interprismatic regions on the basis of a correlation of the appearances seen in different planes of section: these two substances or regions are identical in content and, in fact, in their site of formation (extracellularly). They may only be distinguished with reference to some micro-anatomical feature such as the prism-direction, when it can be seen that they contain the same elements (crystallites) but that these are orientated differently with respect to the prism-direction. (The prism direction is the path described by the TOMES processes of the ameloblasts during the development of a group of prisms). It is not possible to discuss the criteria on which the distinction between "prism" and "interprismatic regions" was made - ie.. from crystallite orientation patterns - without considering the results, when they should be self evident.

This section contains the detailed analysis of the results. Although these are presented as a unified concept, it should be born in mind that they have not been obtained from one method. The finer details of the shape of the "holes" in the "honeycomb", and its relationship to the ameloblasts and the prisms are, however, all derived from the electron-microscopic results.

The developing surface of enamel contains as many half-conical depressions as there are half-conical projections of the ameloblasts (TOMES'processes) filling them. Each ameloblastic process fills one depression, and every ameloblast is in contact with its neighbour just above the depressions (at the level of the inner-end terminal bar apparatus. The presence of the depressions is the direct result of the mode of secretion of the enamel around the TOMES' process projections of the ameloblasts).

The prisms are bundles, or three-dimensional domains of crystallites in which there is only a small change in orientation between neighbouring crystallites or groups of crystallites. The prisms are not related to the individual depressions in the developing surface in the same way as are the ameloblasts. It is an oversimplification to state that a prism represents the territory within the enamel resulting from the filling in of one depression though this is the case in the "round prisms" (with complete, circular prism-sheaths) of the Sirenia In many mammals each prism forms between two ameloblasts e.g. Ungulata, Rodentia, Lagomorpha, or three ameloblasts in some instances in the Primates and Proboscidea. Likewise one ameloblast may be related to material constituting parts of one two or three "prisms" (Patterns, 1 2, and 3; Figs. 1.1, 1.2, and 1.3 respectively).

The depressions in the surface of developing enamel are assymetric: often one side is flat.The depressions do not taper evenly (ie from all sides) to a point, and rarely face perpendicular to the surface: sections having different orientations with respect to the long axis of the tooth will therefore appear different. Three principal planes of section will be described; called "Honeycomb", "Battlements" and "Picket Fence" respectively, and related to the axes of the tooth, ameloblasts and prisms.

back
p.2.14.

Fig. 2.11.A. shows the picket fence or saw-tooth contour of the mineralising front of enamel which is encountered in near-longitudinal sections of developing tooth-crowns. Enamel above; ameloblasts below. The dashes in the upper part of the diagram represent the crystallite orientation in the developing enamel; their length is of no significance.
Left = occlusal, Right = cervical.
One ameloblast sits in each depression in the developing front and each depression is related to two prisms in the development of Pattern 2 enamel (depicted in this diagram).

Fig. 2.11.B. (overleaf) The picket fence appearance is also encountered in near-longitudinal sections of developing Pattern 3 enamels. This diagram shows that the same type of profile results whether or not the plane of section passes through the winged-process regions of Pattern 3 prisms, but that the apparent repeat-distance of the depressions changes radically

FIGURE * 2.11
Longitudinal ("Picket Fence") section through one row of prisms in developing Pattern 2 enamel.

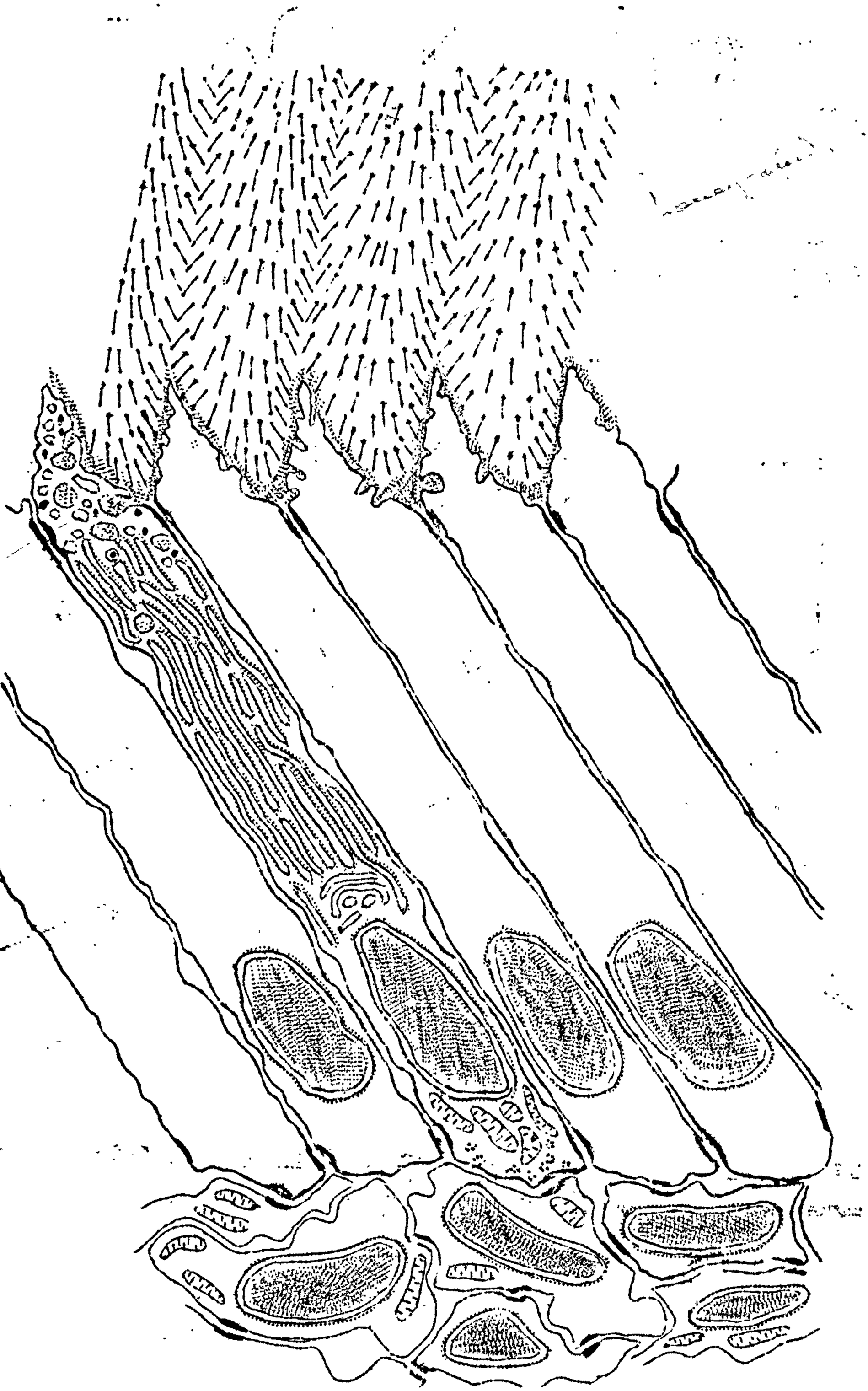


FIGURE 2.11.B.

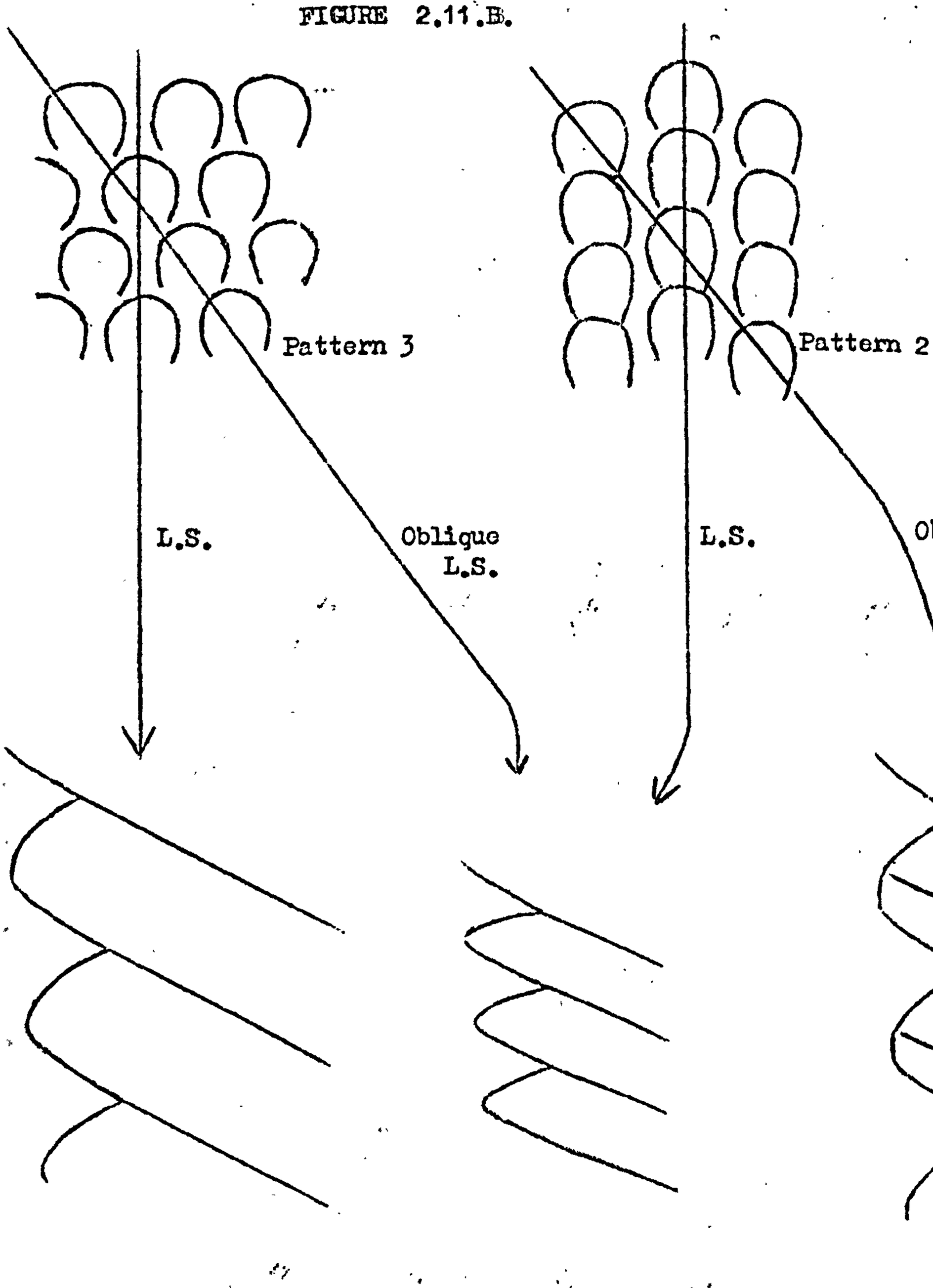


Diagram illustrating planes of section giving rise to "picket fence" profiles of the surface of developing enamels.

2.15 Longitudinal sections of the tooth, prisms and ameloblasts - the "PICKET FENCE" configuration of the developing surface (Diagram, Figure 2:11).

The developing surface displays the well known Picket Fence or saw tooth contour in longitudinal section. The crystallites in the centre of each prism are found to be parallel with the axis of the prism. On either side of this approximate centre they diverge to an increasing extent from the prism axis with increasing distance from the prism centre. At the meeting plane of adjacent prisms, crystallites meet at an angle and the plane at which they meet is the only feature which can be used to define a prism boundary. In the "picket fence" plane of section there is only one discontinuity in the gradual change of crystallite orientation between two "prisms" and there is, therefore, no definable "interprismatic" region.

The innermost point of TOMES' process, or the deepest point of the depression between the spikes of the "Picket Fence", is related to the sharp change of crystallite orientation between the (feather-like arrangements in) adjacent prisms - i.e. the "prism sheath" region. The most prominent points of the developing enamel - the spikes of the picket fence - are related to the regions of attachment between adjacent ameloblasts. The crystallites forming in relation to these points are within the "prisms": they are certainly not in a region which can be defined as "interprismatic" . The crystallites develop (very approximately) perpendicular to the surface of the individual protuberances of the profile of the developing surface (the "pickets" of the fence). These projections are much more rounded off than the description "spike" would suggest, so that there is a gradual change in orientation of the crystallites from one side of each projection, to the other. That this results in the feather-like (repetitive) pattern of orientation of the crystallites seen in the "Picket-Fence" plane of section is obvious from a consideration of Diagram 2.11. (The crystallites which develop in immediate relation to the projections are not parallel to the prism-axes because the prisms are not perpendicular to the developing surface). The sudden change in orientation of the crystallites at

Fig. 2.12.A. (opposite and overleaf) Diagrams showing the planes of section which will give rise to the "Battlements" and other profiles of the surface of developing enamel.

Sections parallel to the transverse axis of the tooth germ but which slope cervically and inwards (parallel with the "prism direction") from the enamel surface give rise to the "Battlements" profile. Sections parallel to the prisms direction but oblique to the transverse axis of the tooth show modified profiles of the surface of the developing enamel.

A "perfect Battlements" section of developing enamel of Patterns 1 or 3 characteristically displays "projections" from the developing surface which are of equal length: the depressions into the surface usually appear to be of unequal depth in developing Pattern 2 enamels.

Fig. 2.12.B. Diagram showing the appearance of the amelobasts in a "Battlements" section of developing Pattern 3 enamel.

Fig. 2.12.C. Diagram showing the appearance of the ameloblasts in a "Battlements" section of developing Pattern 2 enamel.

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p.2.15.
(2.15c)

Diagram Figure 2.12.A

Fig.
2.12.

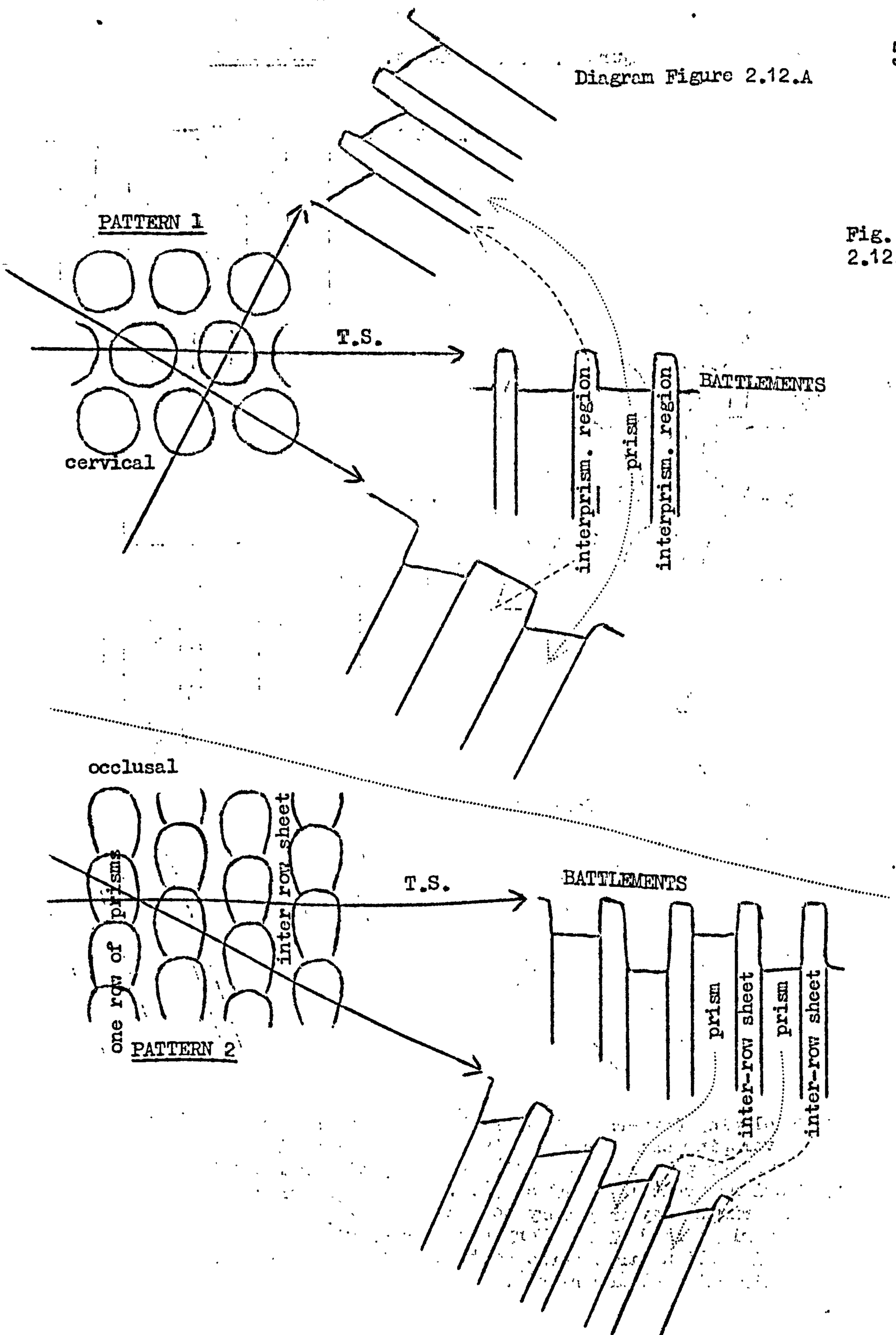
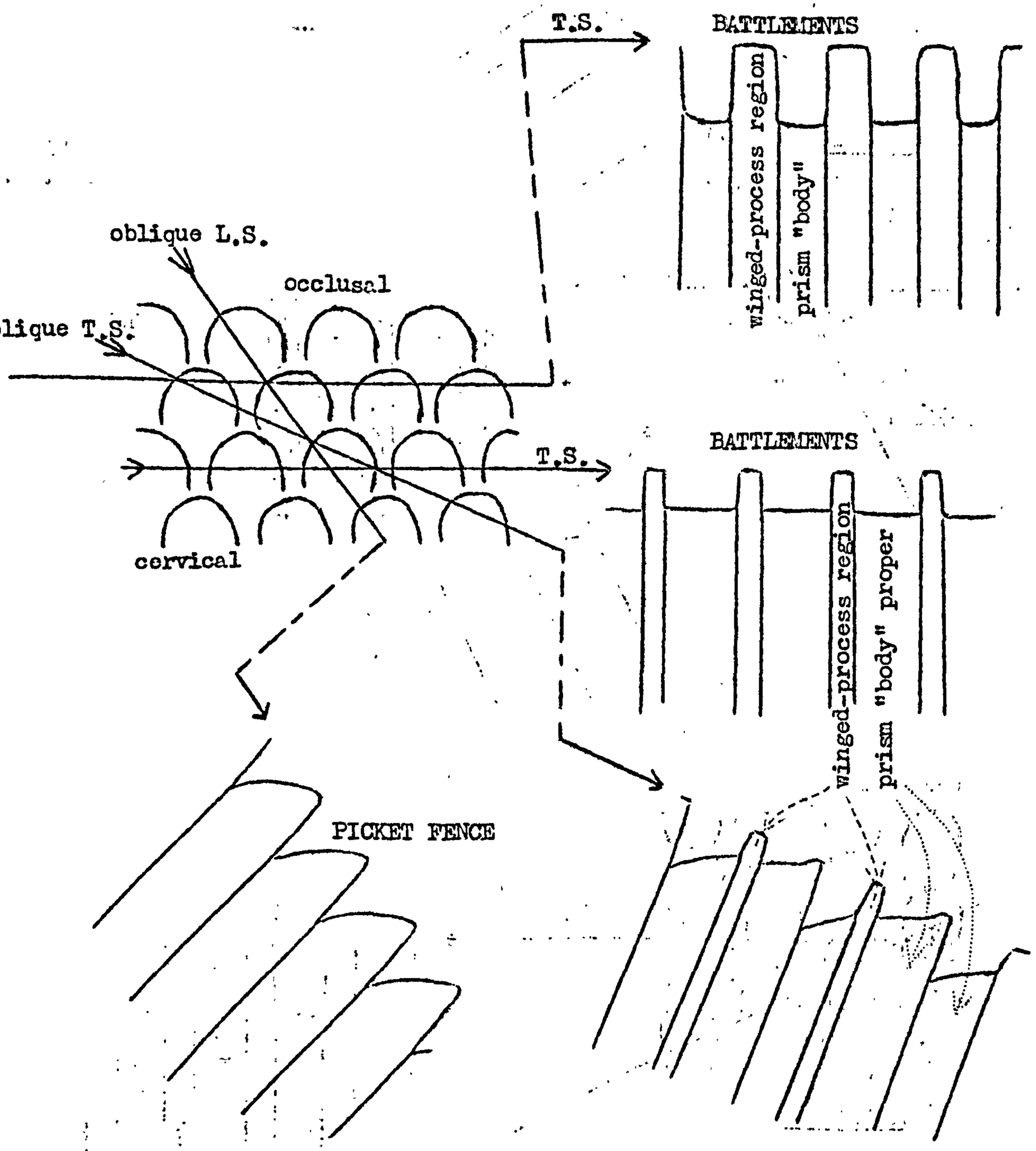


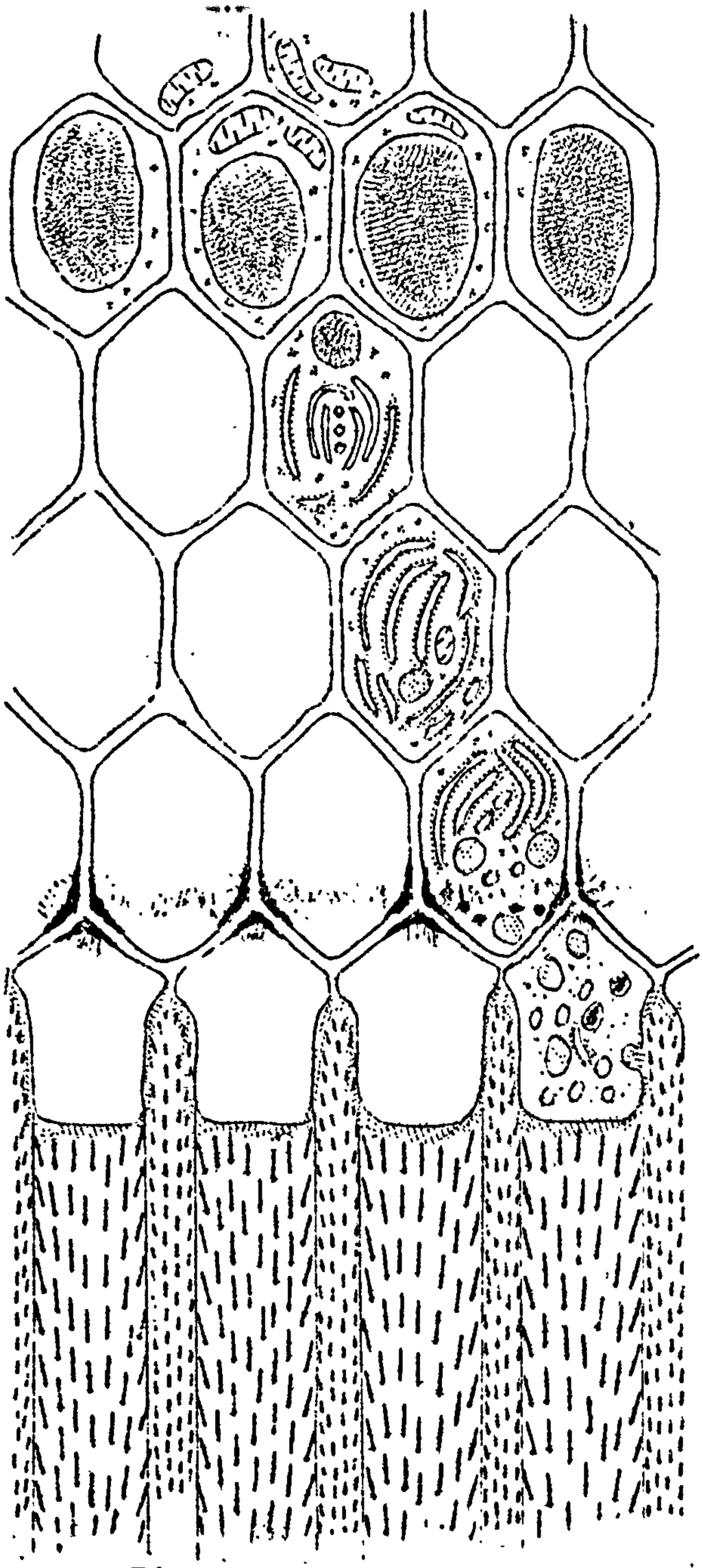
Diagram Figure 2.12.A.continued.



In developing Pattern 3 enamel, the width of the domains within the developing enamel related to the depressions in the surface ("prisms" proper) and the width of the domains related to the "projections from" the surface (i.e. the "winged process" regions of the prisms) may vary very considerably (top and middle diagrams on right hand side). Very peculiar profiles of the surface appear in oblique transverse sections which are parallel to the prism direction (lower right).

2B
15c)

Diagram Figure 2.12.B. PATTERN 3 "BATTLEMENTS"



mitochondriae

nuclei

FIG. 2.12B.

Golgi zones

α-cytomembranes

terminal bar apparatus (inner)

Diagram showing the appearance of the ameloblasts in a "Battlements" sectionof developing Pattern 3 enamel. The prisms are sectioned longitudinally; the ameloblasts were assumed to make an angle of 45° with the prisms. Any line parallel to the surface of the enamel is also parallel to a transverse plane through the tooth.

The narrower fields of more transversely sectioned crystallites (short dashes) are related to the "projections from" the surface of the enamel; they are the winged process regions of their prisms. The wider fields of more longitudinally sectioned crystallites (long dashes) are related to the depressions and are the "prism-body proper" regions of the next (above or below) transverse row of prisms.

Diagram Figure 2.12.C. PATTERN 2 "BATTLEMENTS"

Fig. 2.12C (2.15f back
Fig. 2.12E (2.15

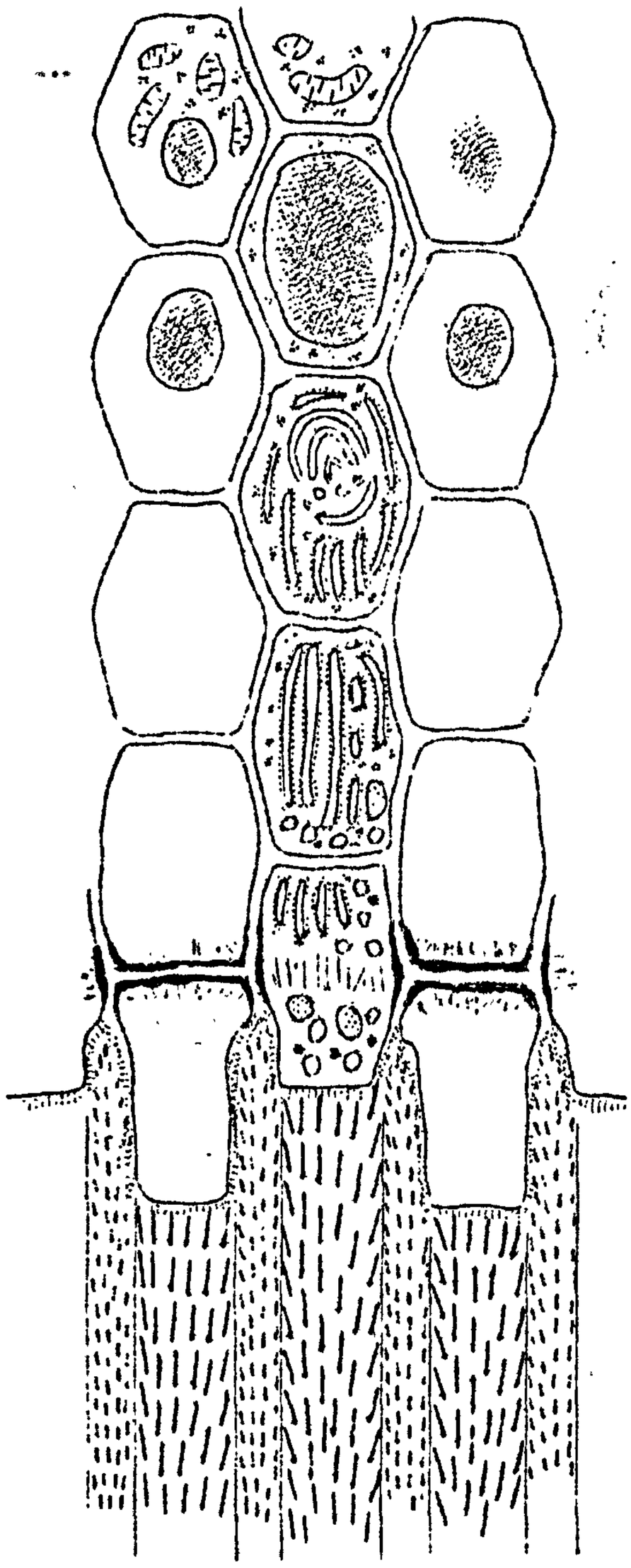


Diagram of "Battlements" section of developing Pattern 2 enamel and its ameloblasts. The ameloblasts were assumed to make an angle of 45° with the prism direction in making this diagram. (This section cuts the prisms longitudinally - see text).

The wider fields of more longitudinally sectioned crystallites (long dashes) are related to the depressions in the surface of the developing enamel; they are "prisms". The narrower fields of more transversely sectioned crystallites (short dashes) are related to the points of the enamel surface most prominent from it; they are inter-row sheet regions.

The depressions belonging to alternate longitudinal rows of prisms appear to be of different depths because they are sectioned at different relative vertical levels.

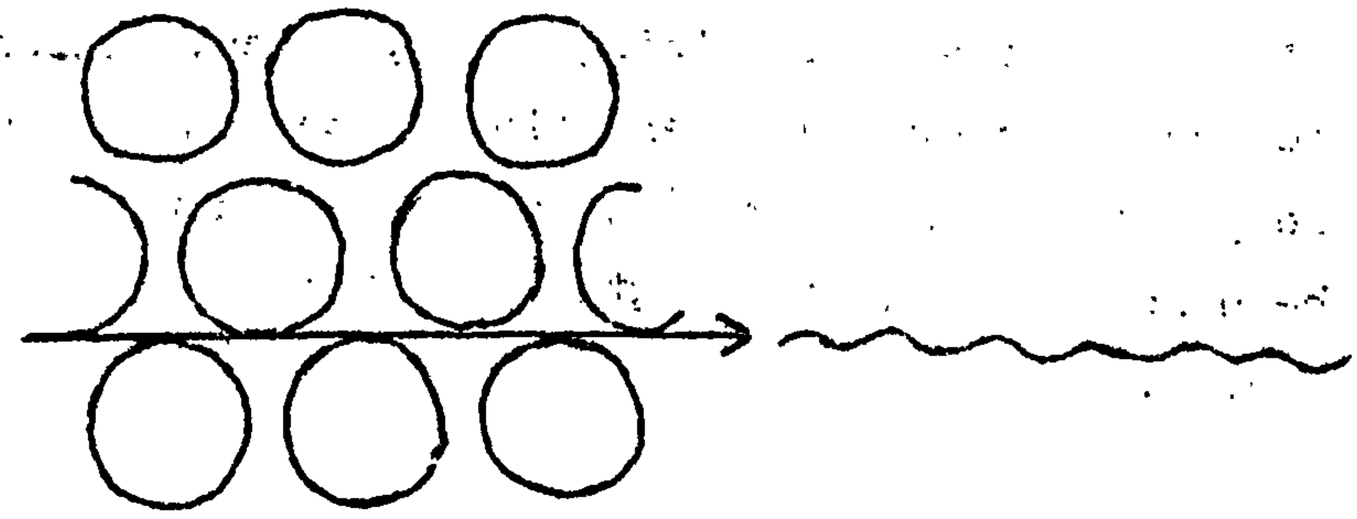
2:16 at the prism -sheath is related to the equally sudden change in orientation of the mineralising front, which, in turn, is related to the sudden change in orientation of the ameloblast cell membrane at the pointed end of TOMES' process. The depth of the depressions in the honeycomb and the length of TOMES' process varies from species to species.

Transverse sections of the tooth, which slope cervically away from the developing surface, present the "BATTLEMENTS" profile of this surface. (Diagram Figure 2,12). A true transverse section of the tooth will not contain the longitudinal axes of the prisms, because the prisms (in lateral enamel) slope away cervically from the surface of the developing enamel. The ideal plane of section under consideration, does section the prisms longitudinally and therefore slopes away cervically from the surface at the same angle as the prisms themselves.

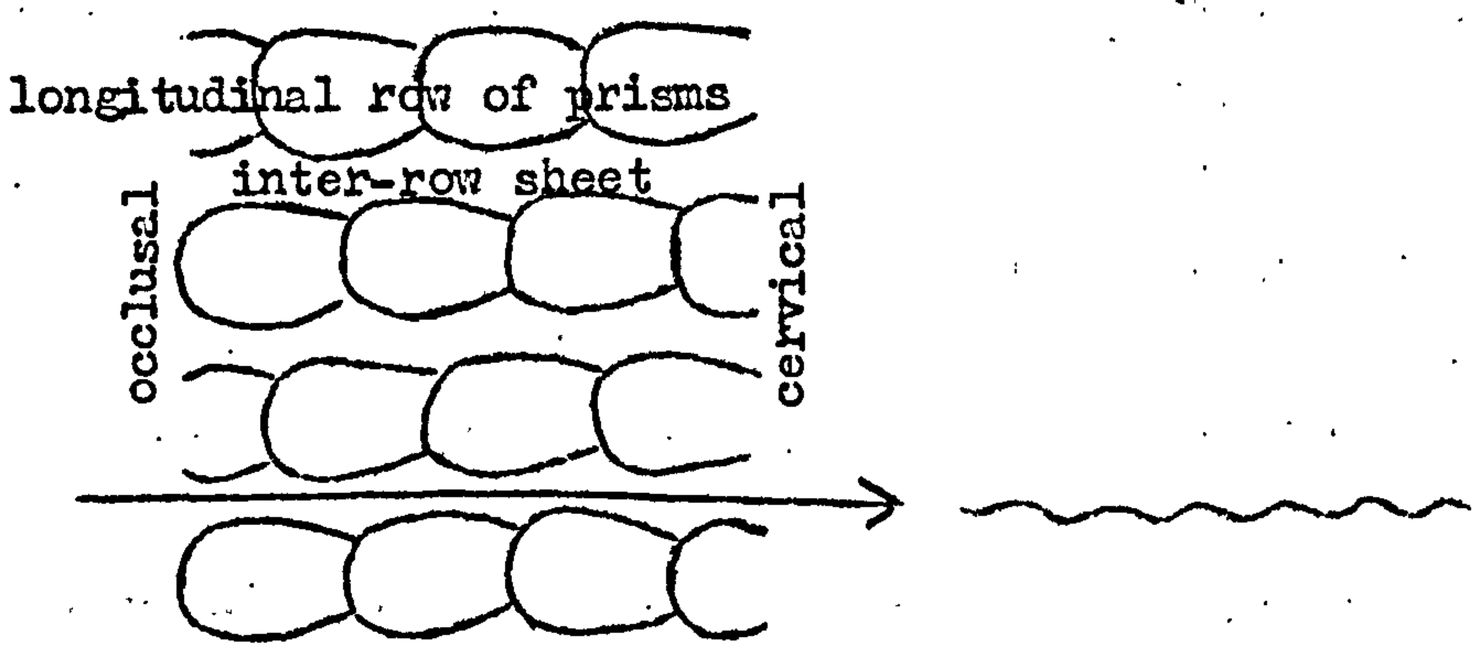
The ameloblasts are sectioned obliquely in this plane, since the prisms normally make an angle of something like 45^{o} with them (see diagram figure 2.11). The developing "enamel" presents a series of narrow, almost oblong projections from its surface and these are reminiscent of the decorative projections from the tops of the walls of mediaeval castles known as battlements.

The crystallite long axes are perpendicular to the mineralising front in the wider domains of more nearly longitudinally sectioned crystallites, i.e. in the "prisms". The narrower fields which contain the shorter fragments of crystallite correspond to the battlement projections - the long axes of the crystallites in these sites (which are either true interprismatic regions, interrow sheets, or winged processes in patterns 1, 2, and 3; Diagram figures 1.1., 1.2., and 1.3. respectively - see later) are nearly parallel with the projections and the mineralising front of the projections. The crystallites within the "prisms" do not deviate in a feather-like fashion from the centre of the prism to anything like the extent seen in the "picket fence" plane of section.

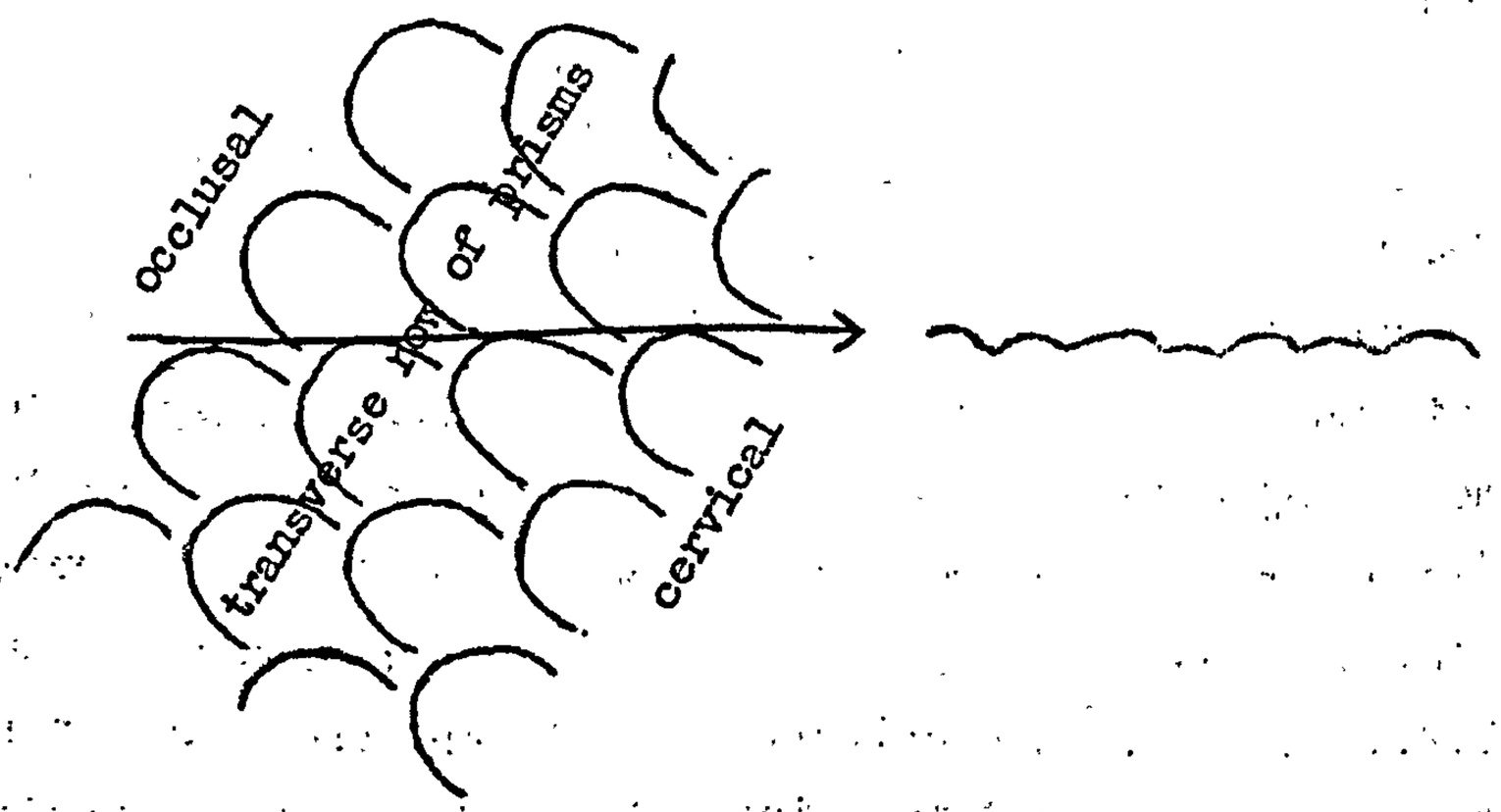
PATTERN 1



PATTERN 2



PATTERN 3



When a section normal to the surface of developing enamel runs through the most prominent parts of that surface, it is possible that the apparent profile of the surface in that section shall have very little amplitude. This applies to all prism patterns.

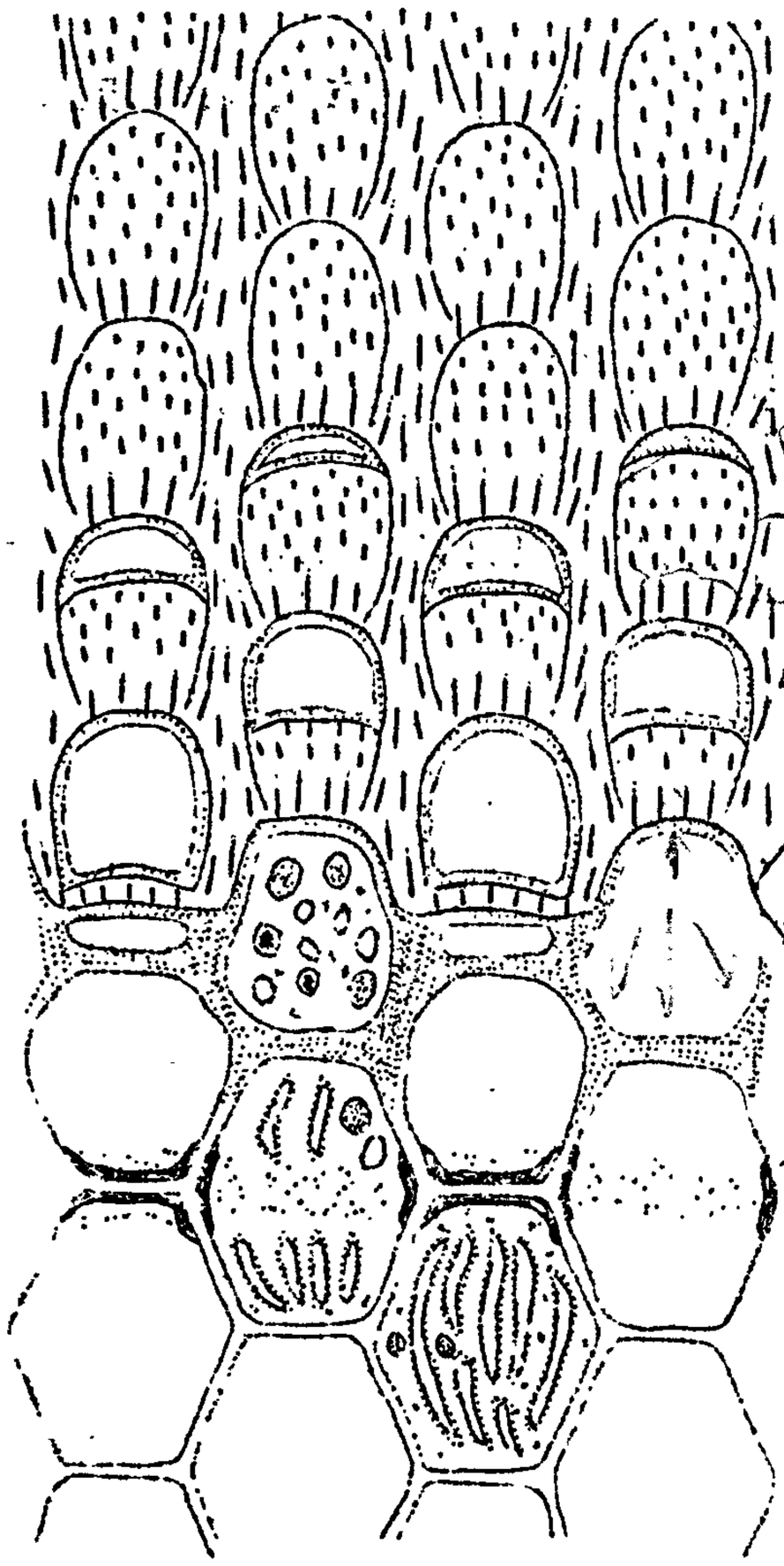


Fig. 2.13.2. PATTERN 2

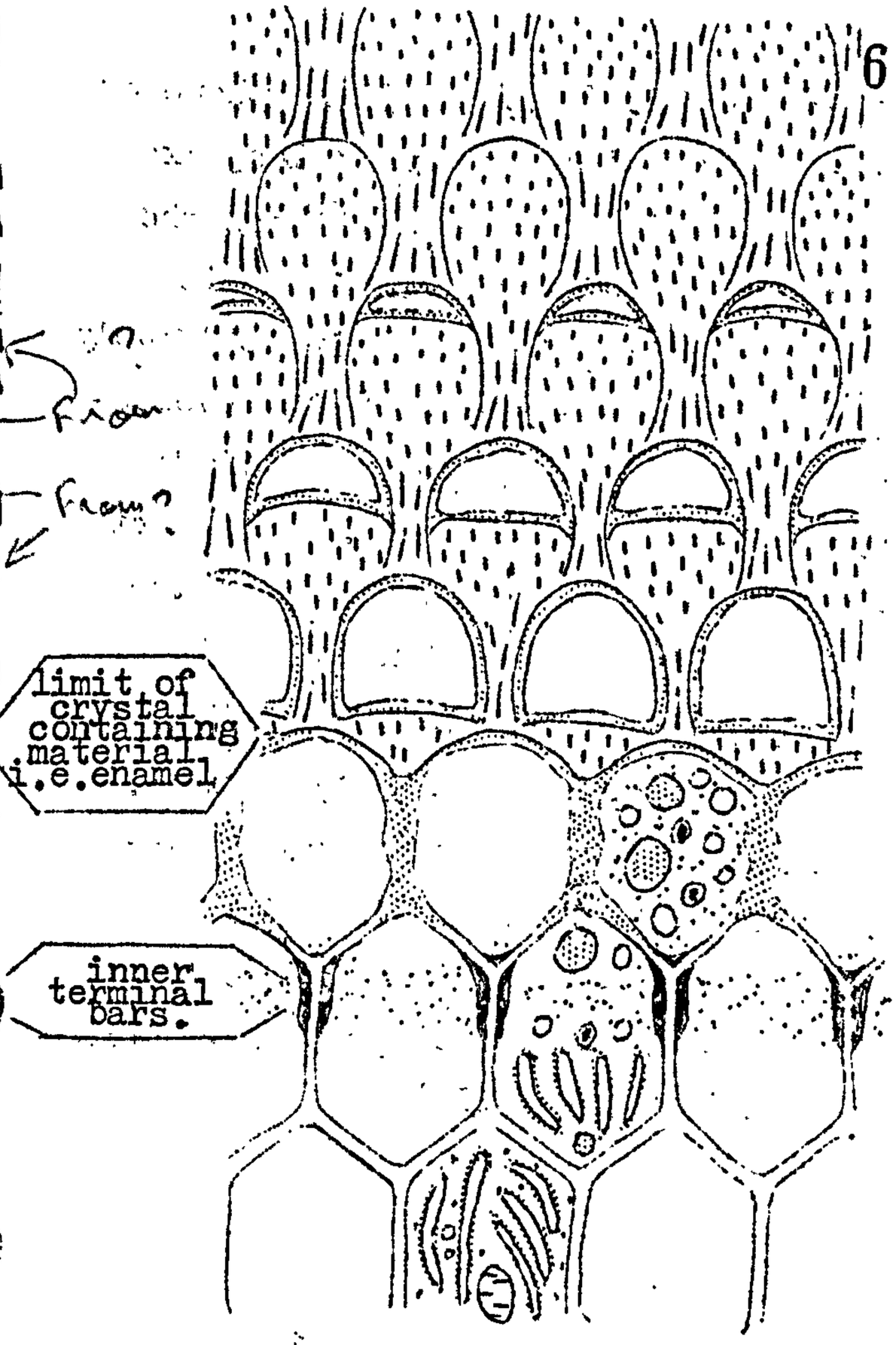


Fig. 2.13.3. PATTERN 3

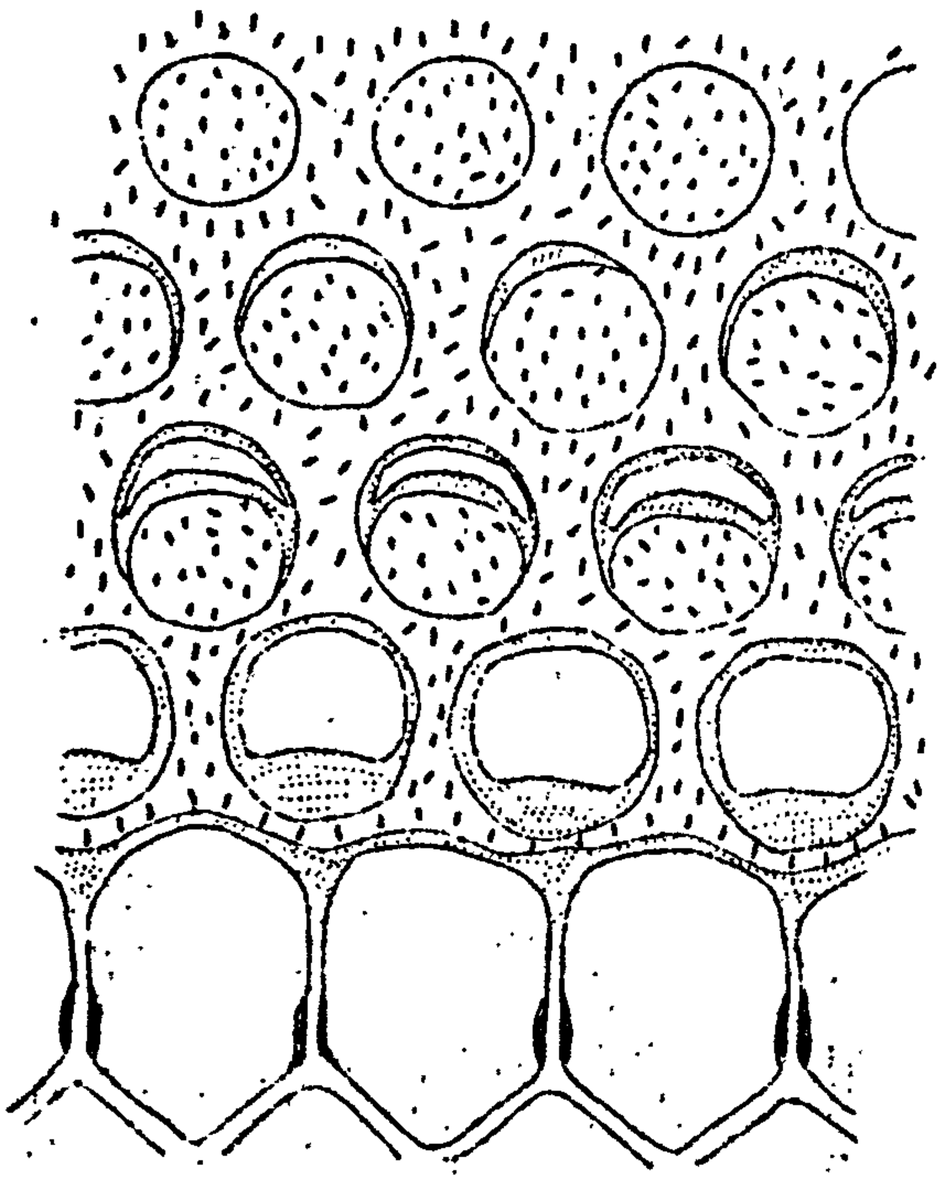


Fig. 2.13.1. PATTERN 1

Figure 2.13. "HONEYCOMB" sections of the surface of developing enamel.

Almost every plane of section which cuts the surface of the developing enamel obliquely will reveal parts of the depressions in this surface occupied by the TOMES' processes of the ameloblasts apparently isolated from the most prominent part of the "enamel". (The important exception is the "Battlements" plane).

In Figs. 2.13.1.,2., and 3 the plane of section cuts the prism direction at 90°, the ameloblasts at 45° and the "enamel" surface at $22\frac{1}{2}^{o}$ from cervical to occlusal. The longest (circumdepression) crystallite fragments lie in true interprismatic regions in Pattern 1 enamel (2.13.1), in inter-row sheet regions in Pattern 2 enamel (2.13.2) and in the winged process regions of Pattern 3 prisms.

In figures 2.13.4., and 5 the plane of section cuts the ameloblast long axes nearly at right angles (78¾° was the angle assumed in constructing this diagram), the prism direction obliquely (56¼°) and the surface of the developing enamel very obliquely (at 11¼°) from occlusal to cervical. The shortest crystallite fragments lie in the prism body proper regions of both PATTERN 3 (Fig. 2.13.4) and PATTERN 2 (Fig. 2.13.5, on the right) enamels. The longer crystallite fragments lie in the occlusal part of the prism bodies and in the inter-row sheet (Pattern 2) and winged process regions (of Pattern 3 enamels)

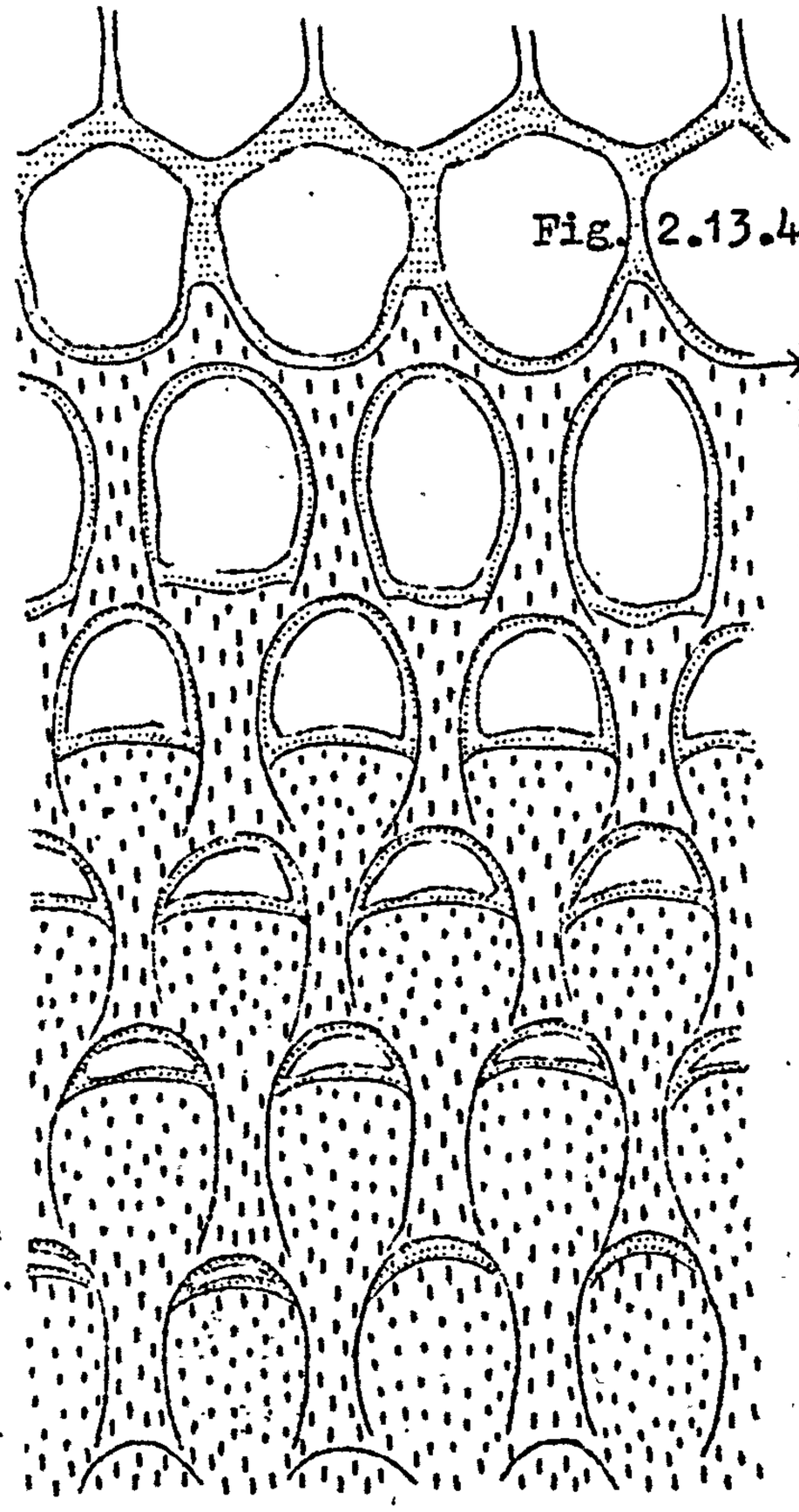


Fig. 2.13.4.

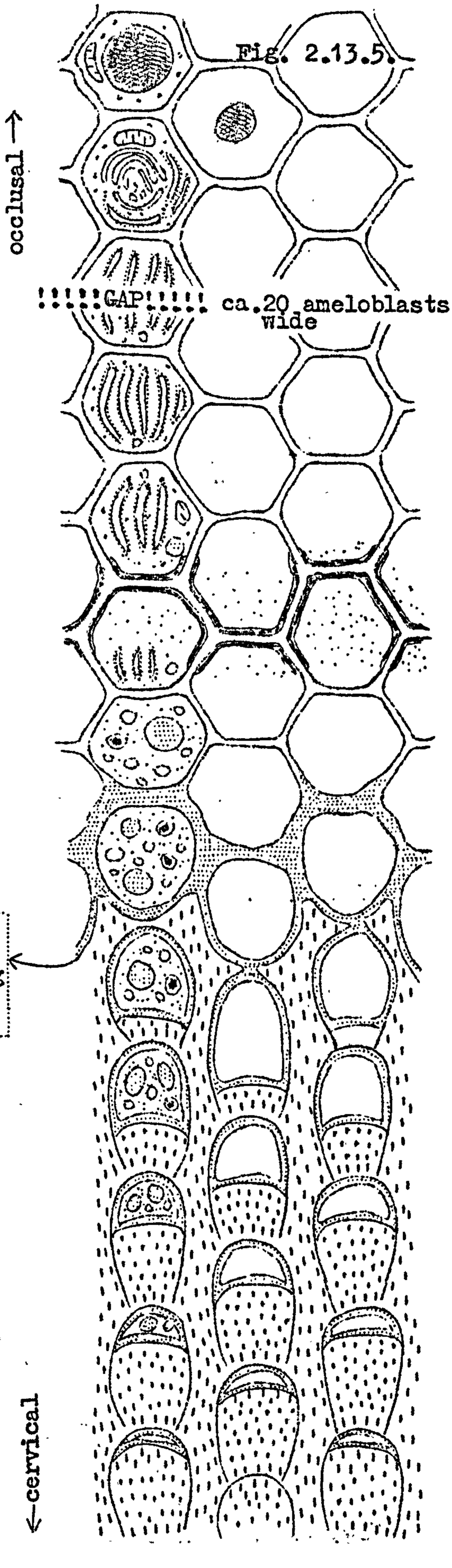


Fig. 2.13.5.

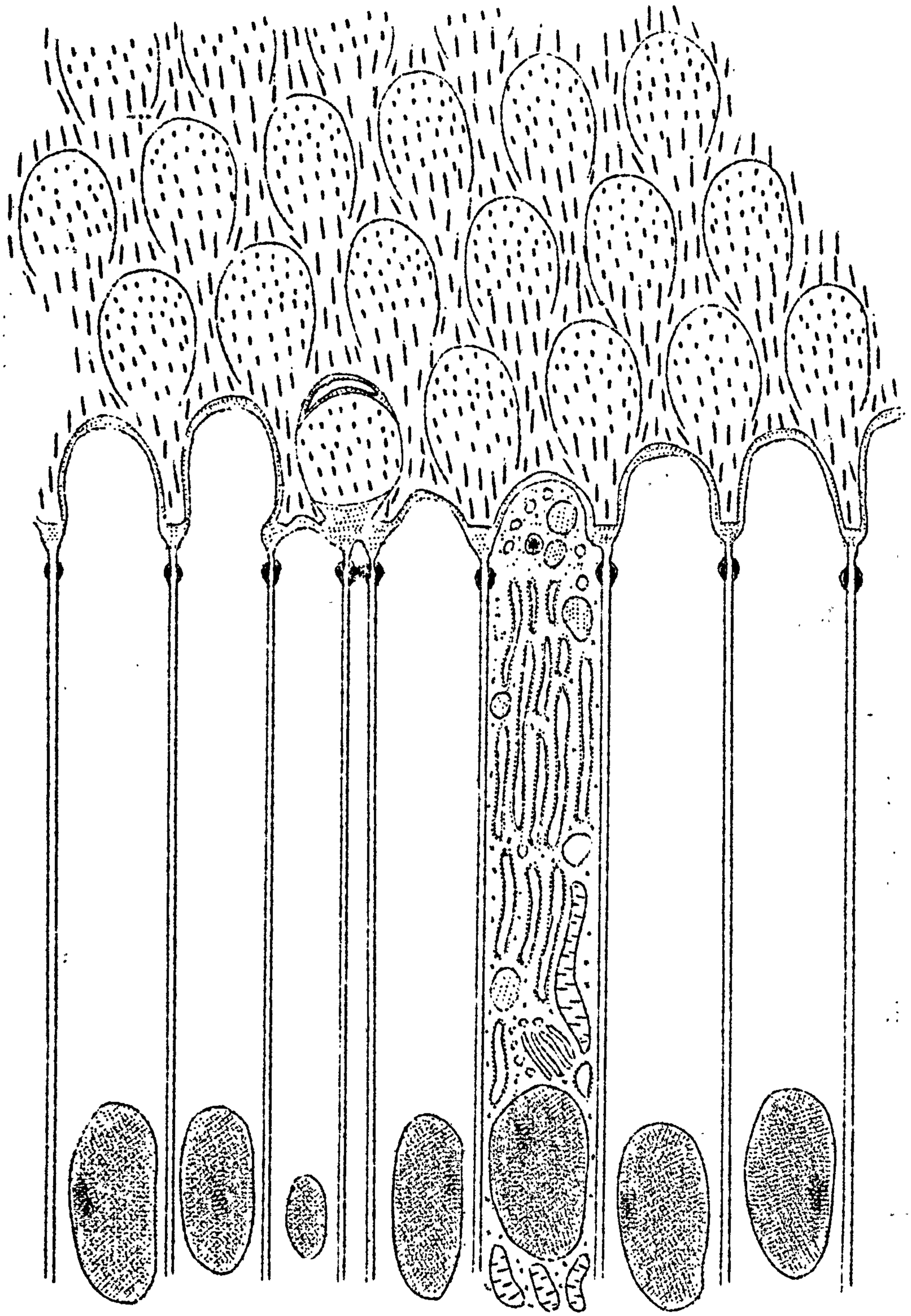


Figure 2.13.6. PATTERN 3

In figs. 2.13.6. (above) and 2.13.7. (overleaf) the plane of section is parallel with the long axes of the ameloblasts and tilted slightly with respect to the transverse plane of the tooth.

Fig. 2.13.6.(PATTERN 3) The plane of section is parallel to the ameloblasts, 45° to the prism direction, and oblique to the transverse plane of the tooth such that it descends one ameloblast width in crossing six ameloblasts in the same transverse row.

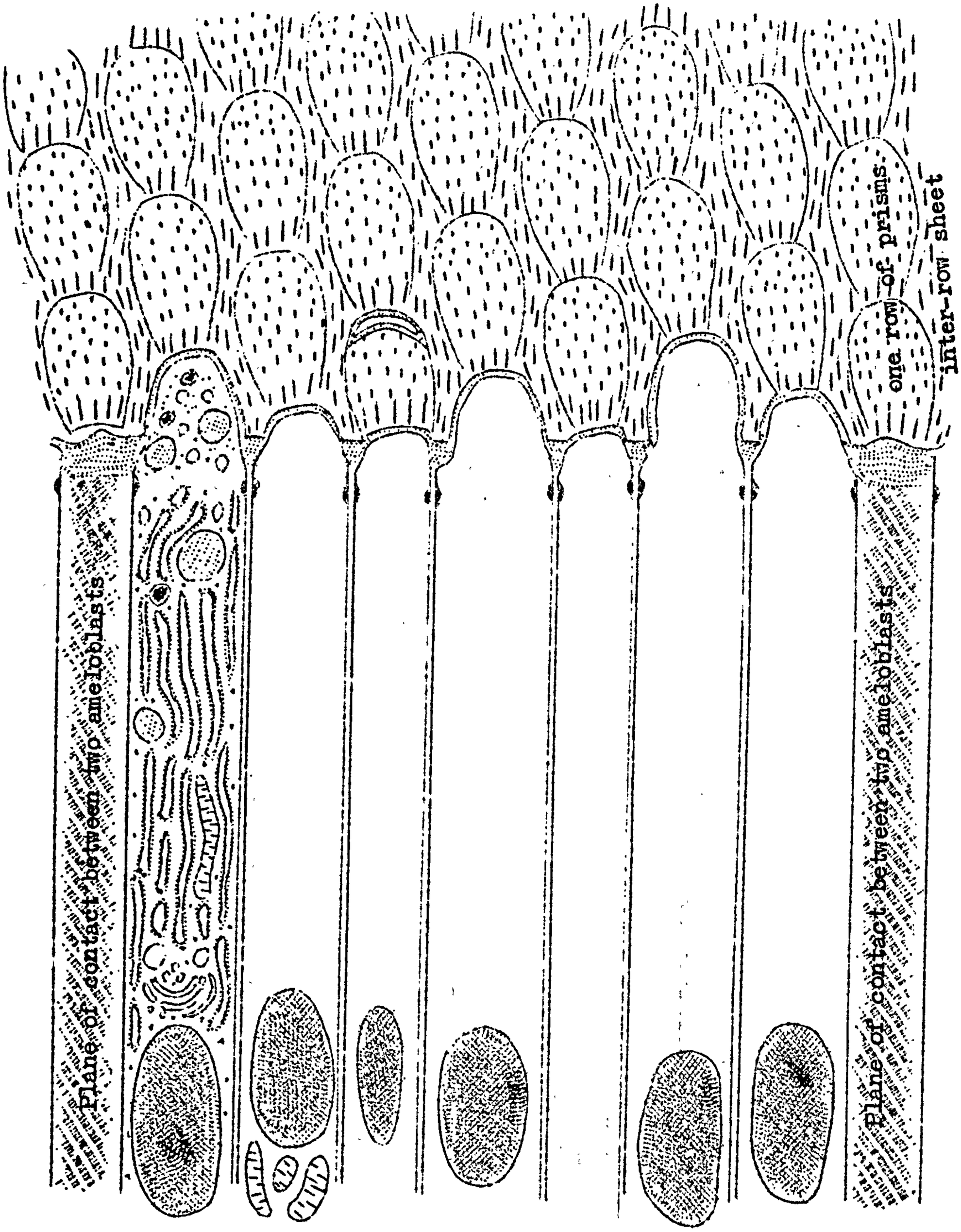


Figure 2.13.7. PATTERN 2

The plane of section is parallel to the ameloblasts, 45° to the prism direction, and oblique to the transverse plane of the tooth such that it descends one ameloblast width in crossing eight longitudinal rows of ameloblasts or prisms.

Even in the plane of section depicted in Figs. 2.13.6., and and 7 it is still possible to find small pieces of TOMES' process cytoplasm apparently separated from the ameloblast cell bodies by the surface of the developing enamel.

2.17

The "HONEYCOMB" configuration of the surface of developing enamel is characteristic of near-tangential sections of this surface. This plane of section may cut the ameloblasts nearly transversely if it enters the enamel from occlusal to cervical.

Sections cut at a tangent to the developing enamel surface pass through the inner ends of the ameloblasts; their terminal bars; the TOMES' processes and the adjacent "Honeycomb" of crystal-containing enamel; and finally the filled-in honeycomb structure.

The "granular material" only appears in an extra-cellular position below the level of the terminal bars and lies between the eell membrane of TOMES' process and the "enamel" (i.e. those regions which contain apatite crystallites). From the pattern seen in this plane of section it can be determined that one ameloblast sits in every hole in the honeycomb of most recently formed enamel. The honeycomb of enamel, then, develops in an inter TOMES process (intercellular in a limited sense) position, but to jump to the conclusion that it represents the interprismatic regions of the adult enamel is only justified in the case of certain prism arrangements (Pattern 1, figure 1.1. and Pattern 2, interrow sheet regions only)

Transverse sections of the prisms can be recognised as such because of the existence of the "prism-sheaths" (because there is a sudden change in crystallite orientation at these planes). The crystallites which fill in the depressions in the surface of developing enamel (intra-depression crystallites) and which are well within the domains which can be defined as prisms are all sectioned nearly transversely; the difference between the length of the fragments of sectioned crystallite is not very noticeable. The crystallites which at one time formed the walls of the depressions in the mineralising front (interdepression crystallites) are also sectioned nearly transversely in Pattern 1 (Figures 1.1. and 2.13.1.) where they lie in true interprismatic regions, but may be sectioned more obliquely in Pattern 3 (Fig. 1.3. and 2.13.3.) where they lie in the "winged process" regions of arcade-formed prisms and even almost longitudinally in the interrow sheets in Pattern 2 (Figures 1.2 and 2.13.2)

2.18

The process of filling in the holes in the basically hexagonal continuum of the first formed enamel occurs most rapidly from one side (figure 2.26— except in Pattern 1 enamel with cylindrical complete prism boundaries) and that one side is destined to be the open side of the "horseshoe" or "arcade" prisms. From this side in, towards the prism body, there is no break in the gradual change of crystallite orientation. When the filling-in reaches the other sides of the prisms (which have been "growing" more slowly) the line of junction between the material within the prism and that belonging to the original honeycomb, is a line of junction between domains containing crystallites having different orientations and it persists as the "prism-sheath". The prism sheath is, therefore, structurally a plane of abrupt change of crystallite orientation and is developmentally related to the mode in which the depressions in the mineralising front fill in.

It is difficult to determine what proportion of the growth of enamel in the holes (depressions) in the honeycomb occurs from the different sides of these holes. However, it is certain that it occurs to some extent from all sides, since the walls of the holes in the honeycomb at a level nearer to the terminal bars are narrower than at that level at which the final filling up is achieved. Each ameloblast contributes material to: 1) one prism and the true interprismatic region surrounding it in Pattern 1 enamel (Figures1.1 and 2.13.1):2)two prisms and the adjacent interrow sheet interprismatic region in Pattern 2 enamel (Figures 1.2 and 2.13.2); or 3) three prisms (and a diminutive area of a fourth, Fig. 2.25) in Pattern 3 enamel.

The most prominent enamel is not all destined to be interprismatic in situation, except in the case of enamels with complete, cylindrical (circular transverse-sectional shape) prism boundaries (Sirenia, Odontoceti, Insectivora, Cheiroptera, human cuspal enamel). The most prominent enamel is also interprismatic in the battlements plane of section of longitudinal rows of prisms with interrow sheets of parallel orientated crystals (Pattern 2 enamel). In longitudinal sections through the middle of a longitudinal row of prisms the most prominent enamel is within the prism. In longitudinal sections through Pattern 3 enamel the most prominent enamel is in the "winged process" (MUMMERY, 1919) region.

Figure 2.14. Scanning electron micrographs of the surface of the developing enamel in various mammals. back 2.18

Fig. 2.14.1. Rhesus macacus (X 400) showing the openings of the depressions in the surface of the developing enamel facing in different directions in groups.

Fig. 2.14.2. Trichecus latirostris (X 1300): the depressions all face in the same direction.

Fig. 2.14.3. Rhesus macacus (X 4000)

Fig. 2.14.4. Myocastor coypus (X 1300): the much narrower depressions match those of the reconstruction of the outer enamel of this species shown in Fig. 2.15.7. It is also possible that the surface was damaged in preparing this specimen.

Figure 2.15. Photographs of wax reconstructions of the surface of the developing enamel in various mammals.

Fig. 2.15.1. Rat incisor inner-enamel. The matches are inserted in the depressions in the surface of the developing enamel (i.e. the wax) to indicate the direction which these holes (which represent the sites occupied by the TOMES' processes of the ameloblasts) take with respect to the surface. The alternate rows of holes (matches) make angles of 90^{o} with each other and lean at 45^{o} to the surface. Incisal to the left.

Fig. 2.15.2 Didelphis enamel. The reconstruction was prepared from "Honeycomb" sections. Cuspal to the top.

Fig. 2.15.3. Negative of 2.15.4. The projections from this model represent the TOMES' processes of the ameloblasts.

Fig. 2.15.4. Rhesus monkey permanent molar enamel. Reconstruction prepared from transverse sections of the tooth. Cuspal to the top.

Fig. 2.15.5. Rhesus monkey permanent molar enamel. Reconstruction prepared from longitudinal ("Picket fence") sections of the tooth. Cuspal to the top.

Figures 2.15.6 and 2.15.7. are on the next plate.

Fig. 2.15.6. Coypu incisor inner-enamel. This reconstruction is of interest because it shows the roof shaped end of the E/M block: there was thus no doubt about the way in which the sections (i.e. the wax sheets) should be laid together to make the model.

Fig. 2.15.7. Coypu incisor enamel. The region reconstructed involves the transitional zone between the inner- and outer- enamels. The pins indicate the direction of the "holes" in the developing enamel (i.e. the wax).

The magnification of the above reconstructions is the magnification of the projection microscope used to make the drawings on which the cut-out shape of the wax sheets was based: this was approximately 1500X: the size of the models is only slightly reduced in these photographs. The drawings for Fig. 2.15.1. were magnified X2 (i.e. Total Mag^{n}. of Fig. 2.15.1 = 2500 approx.)

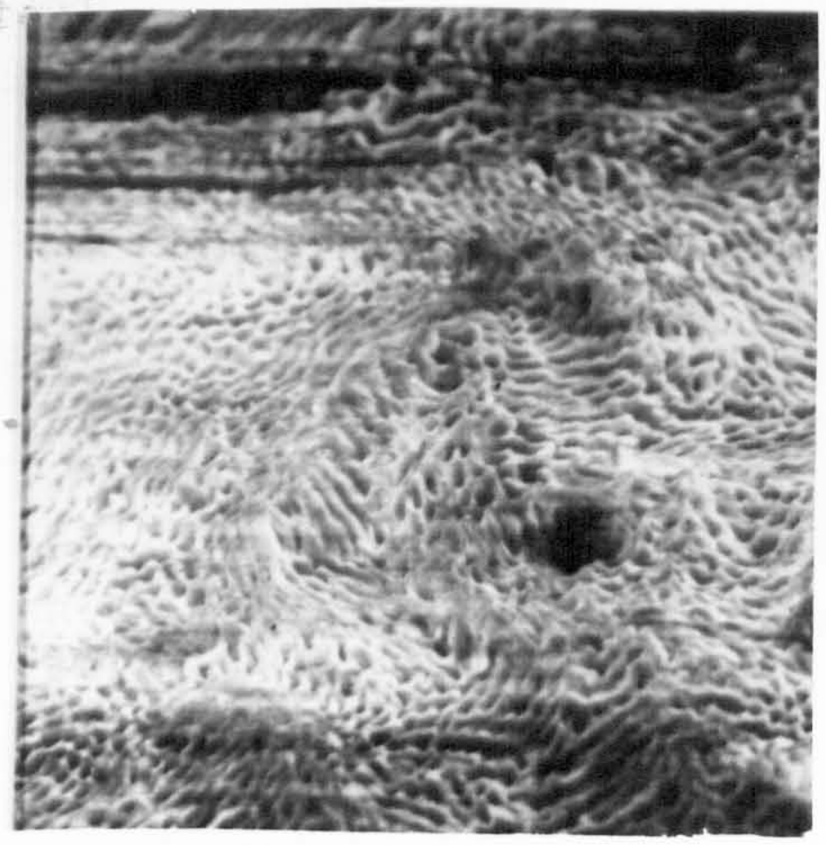


Fig. 2.14.1.
Rhesus macacus (X 400)

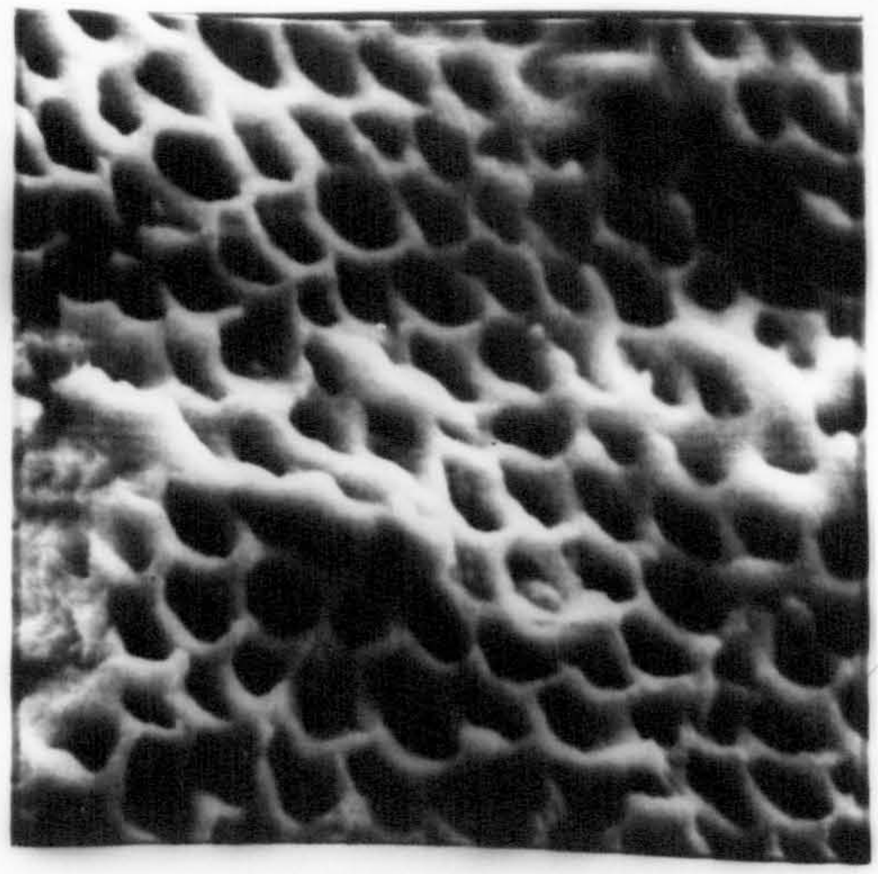


Fig. 2.14.2.
Trichecus latirostris
(X 1300)

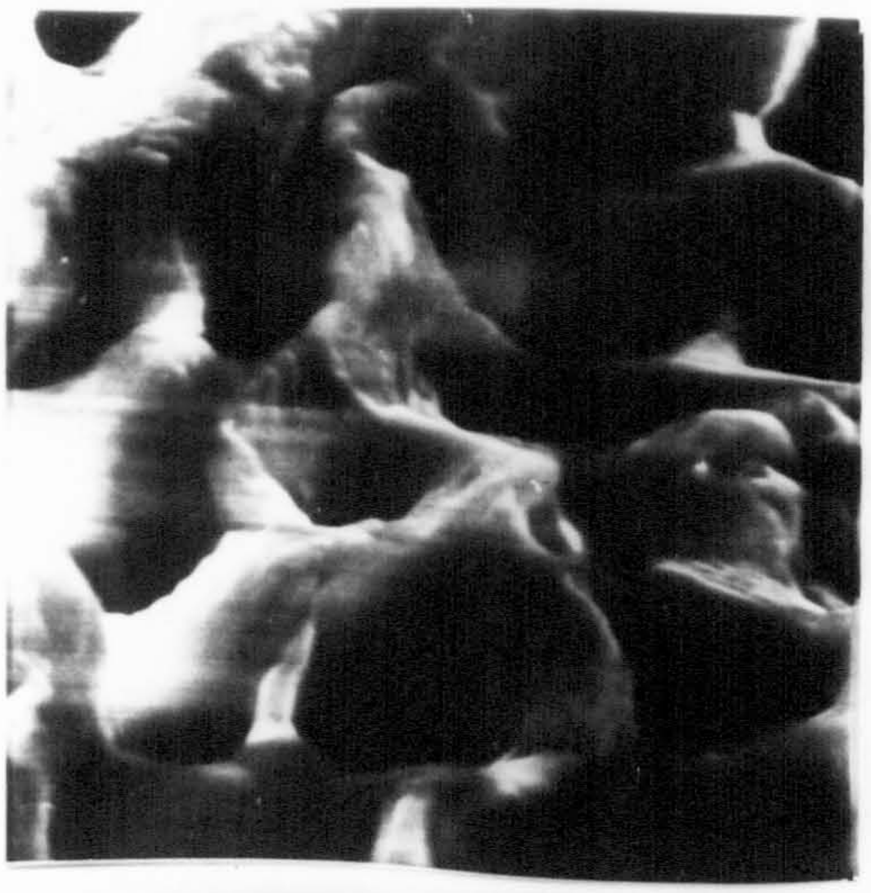


Fig. 2.14.3.
Rhesus macacus (X 4000)

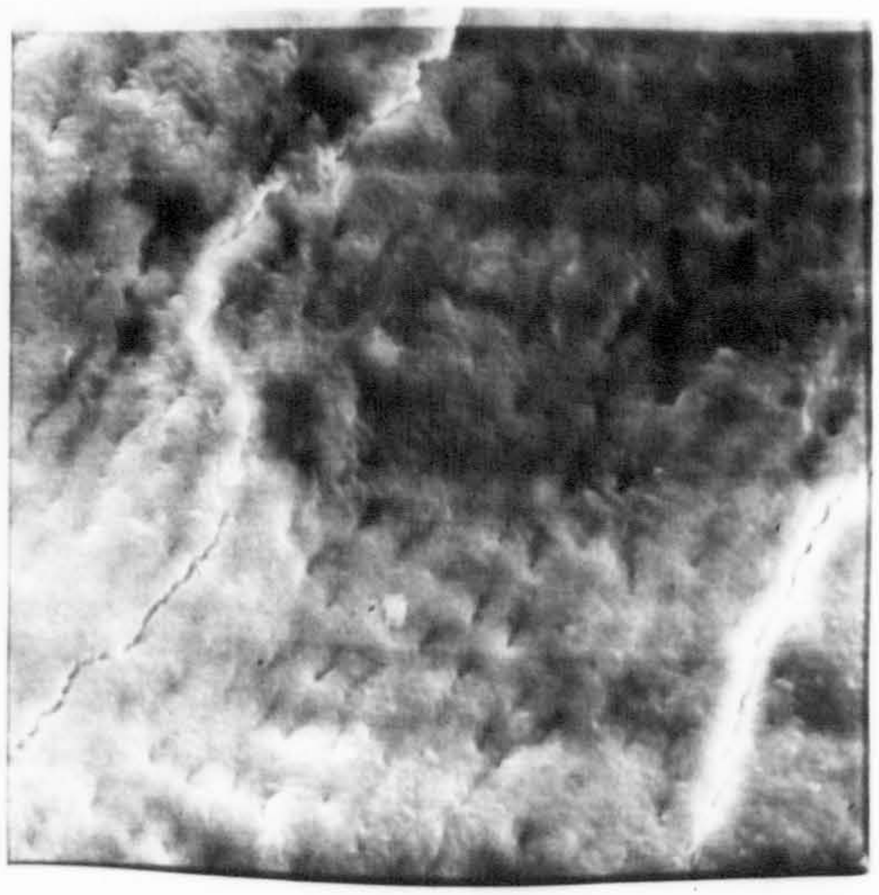


Fig. 2.14.4.
Myocastor coypus (X 1300)

Figure 2.14. Scanning electron micrographs of the surface of the developing enamel in various mammals.

Fig. 2.15.1 Rattus

Fig. 2.15.3 (above) and 2.15.4 (below) Rhesus

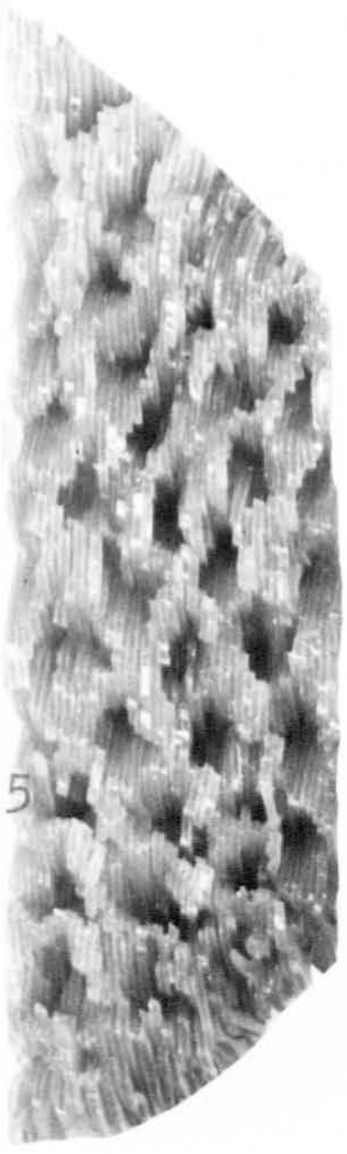


Fig. 2.15.5
Rhesus

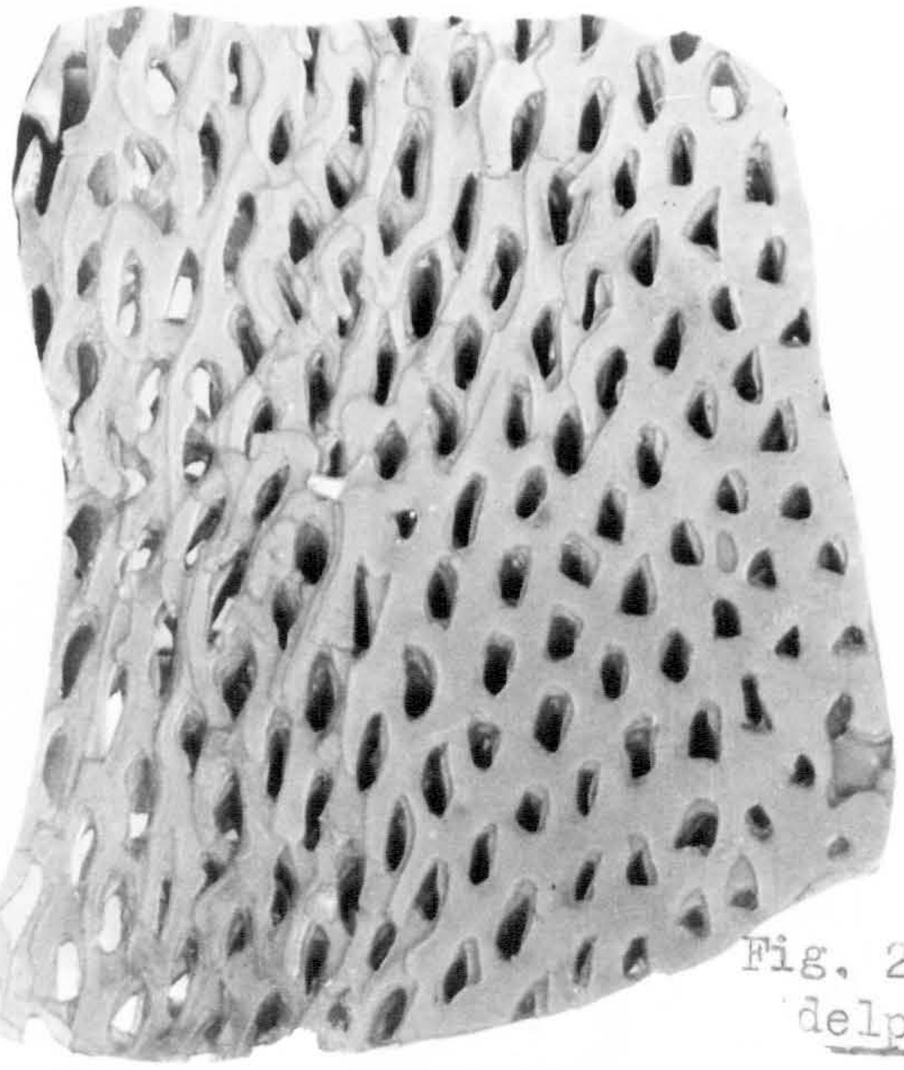


Fig. 2.15.2
delphis

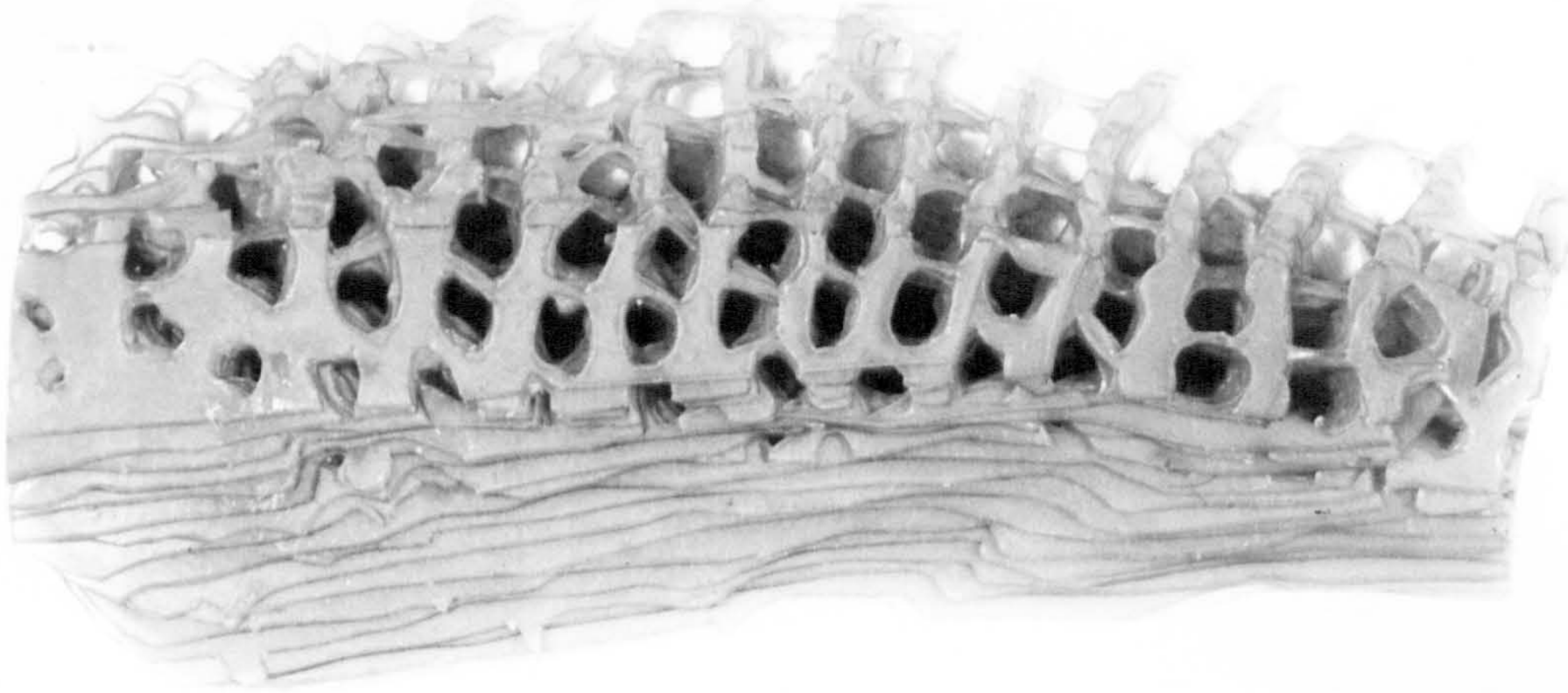


Fig. 2.15.

Figure 2.15.6. Myocastor coypus: roof-shaped end of E/M block.

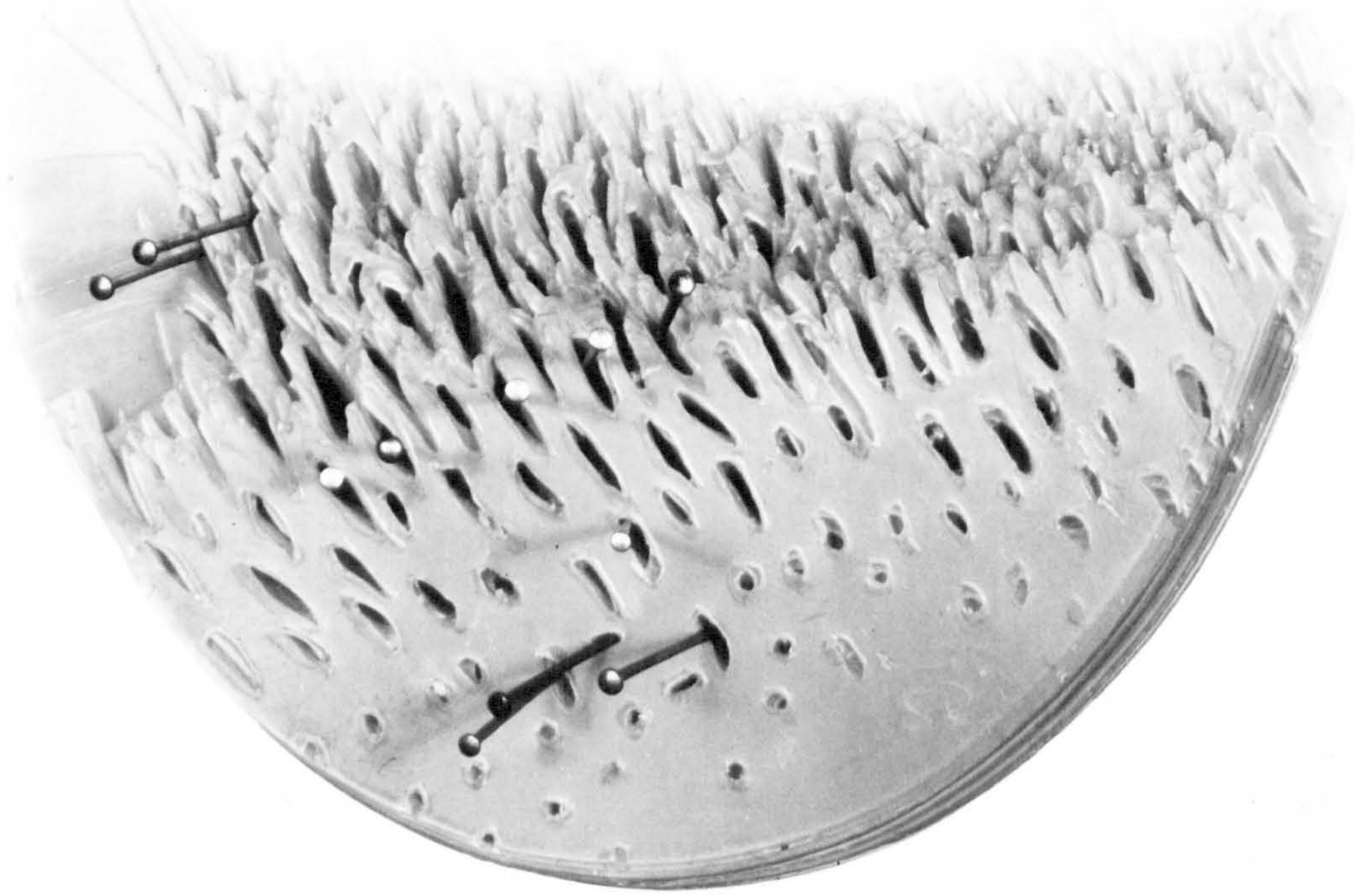


Figure 2.15.7. Myocastor coypus: pins indicate direction of "holes" in the developing enamel (i.e. the wax).

2.19 2.4.2.4. Observations relating to the development of the decussation of prisms or zone formation - HUNTER-SCHREGER BANDS.

Scanning electron microscopy- An impression reminiscent of the HUNTER-SCHREGER bands is given in the low-power micrographs of developing Rhesus macacus enamel in Figure 2.14.1. The openings of the "cells" of the honeycomb are evidently facing in different directions in groups. In the manatee (Trichecus latirostris), however, all the openings of the holes previously occupied by the TOMES' processes of the ameloblasts face in the same direction (Fig. 2.14.2). This difference between manatee and monkey enamel is probably related to the absence of any (noticeable) decussation of the prisms in the former species. The surface of the developing coypu enamel (Fig. 2.14.4) showed a rather disappointing lack of detail; probably because the surface had been damaged during preparation.

Wax reconstructions - some of the most interesting evidence to be derived from these reconstructions came from those of developing rat incisor inner-enamel. In Figure 2.15.1. one can see the direction taken by the matches inserted in the (very long) holes of the honeycomb: imagine the end of the match in the wax to be the TOMES' process of the ameloblast. Alternate rows of matches cross each other at 90°: they lean at 45° to the surface of the wax in opposite directions in alternate rows.

Light microscopy of "conventional histology" material: A "zoning" of goat ameloblasts, occuring at the same sort of interval as the HUNTER-SCHREGER bands zoning, and which may be related to the latter, has been observed. (Fig. 2.16). It is difficult to describe that feature of the arrangement of the ameloblasts which enables these zones to be discerned and it can only be seen at low magnifications. It would be better studied in connection with the development of an enamel with sharply bordered zones, such as occurs in the carnivora. I (regretably) did not prepare suitable material.

Electron microscopy of ultra-thin sections- Rat incisor inner-enamel served best to illustrate the mode of development of the decussation of the prisms (i.e. Zone, or HUNTER-SCHREGER Band formation). Alternate transverse rows of prisms could be seen to fill from alternate though not exactly opposite sides (Fig. 2.17).

Fig. 2.16. Photomicrograph (X 250) of a Haematoxylin and Eosin stained section (cut at 15μ) of a decalcified, celloidin embedded goat molar tooth germ. The (longitudinal) section cuts the surface of the developing enamel nearly tangentially, thus sectioning both the prisms and the ameloblasts nearly transversely. The alternate zones of prisms which lay more nearly normal to the plane of section can be seen to be organised as well ordered longitudinal rows. The enamel-organ cells (ameloblasts and stratum intermedium cells) are also organised into zones which correspond to the zones of prisms in the enamel.

Fig. 2.17. Electron micrograph (X 3460) of a near-tangential section of the surface of developing rat incisor inner-enamel, showing the alternate transverse rows (WSW to ENE) of prisms filling in from alternate sides (i.e. from SSW to NNE and from ESE to WNW respectively). The wavy ribbons of "interprismatic" material running from NNW (incisal) to SSE (apical) in this image are equivalent to the inter(longitudinal) prism row sheets in ungulate enamel. Note the way in which the terminal bar apparatus delineates the transverse rows of ameloblasts.

Fig. 2.18. Electron micrographs of transverse sections of prisms in developing ungulate enamel, showing organisation into longitudinal rows of prisms and inter-row sheets of parallel oriented crystallites.
Figs. 2.18 A and B. Pig enamel (X 1050).
Fig. 2.18C. Calf enamel (X 12600).

Fig. 2.19. Electron micrograph (X 19900) of "Battlements" plane section of developing pig enamel. The wider fields of more longitudinally sectioned crystallites are "prisms"; the narrower fields of more transversely sectioned crystallites are inter-row sheets. The bands of variation in electron density running from left to right across this image are "knife marks".

Fig. 2.20. Photomicrograph (X 1500) of Haematoxylin and Eosin stained section of decalcified, developing Bennett's wallaby incisor enamel, showing organisation into longitudinal rows of prisms. The intense dark specks are the "fibres" of TOMES, i.e. the organic contents of the enamel "tubules".

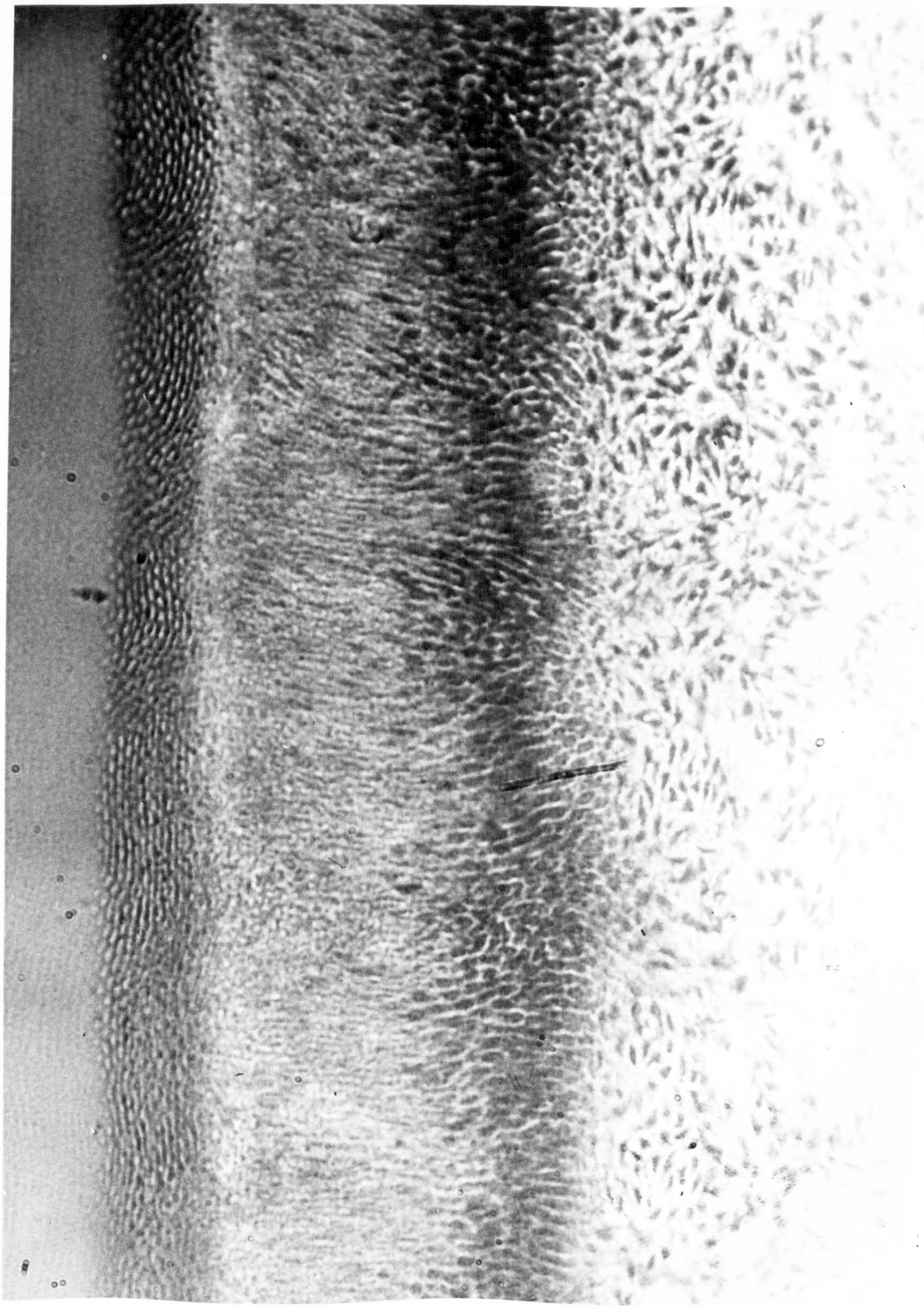


Fig. 2.16

Figure 2.16. Capra: L.S. developing tooth; nearly T.S. prisms and ameloblasts. (X 250).

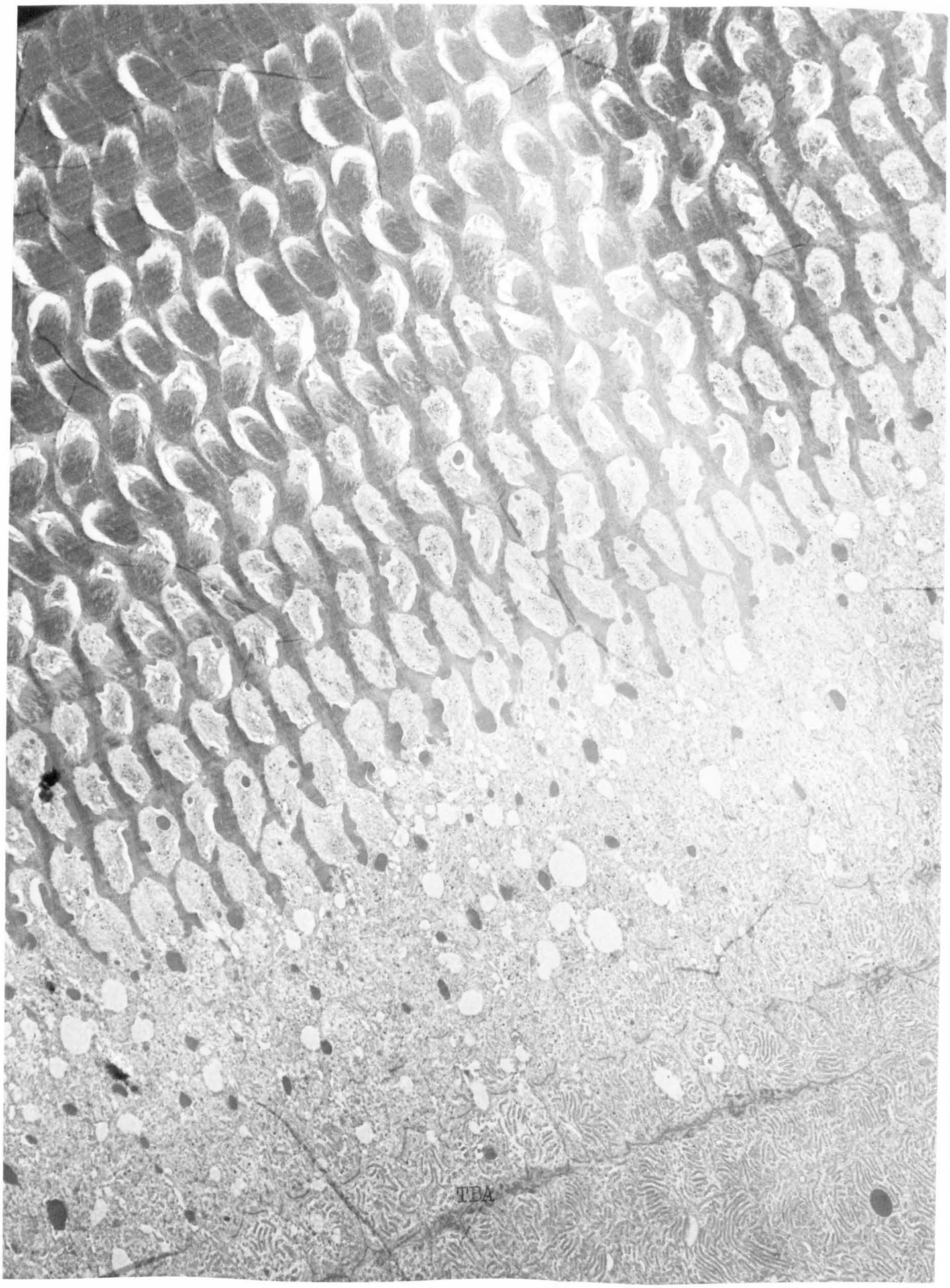


Figure 2.17. Rattus: rows of prisms filling in from alternate sides.
(X 3460)

Figure 2.18. Ungulate enamels. T.S. prisms.

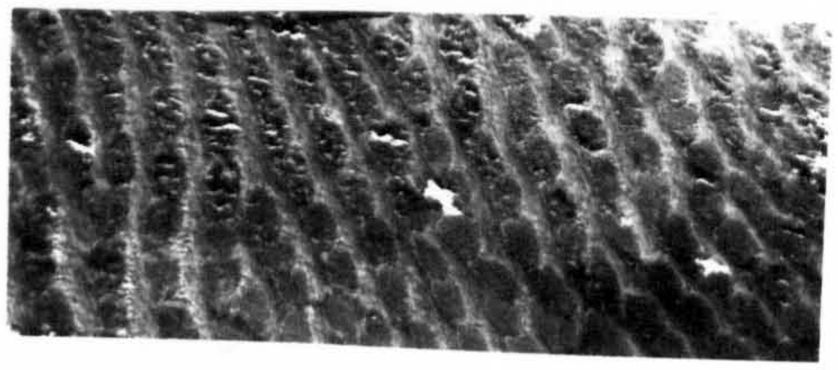
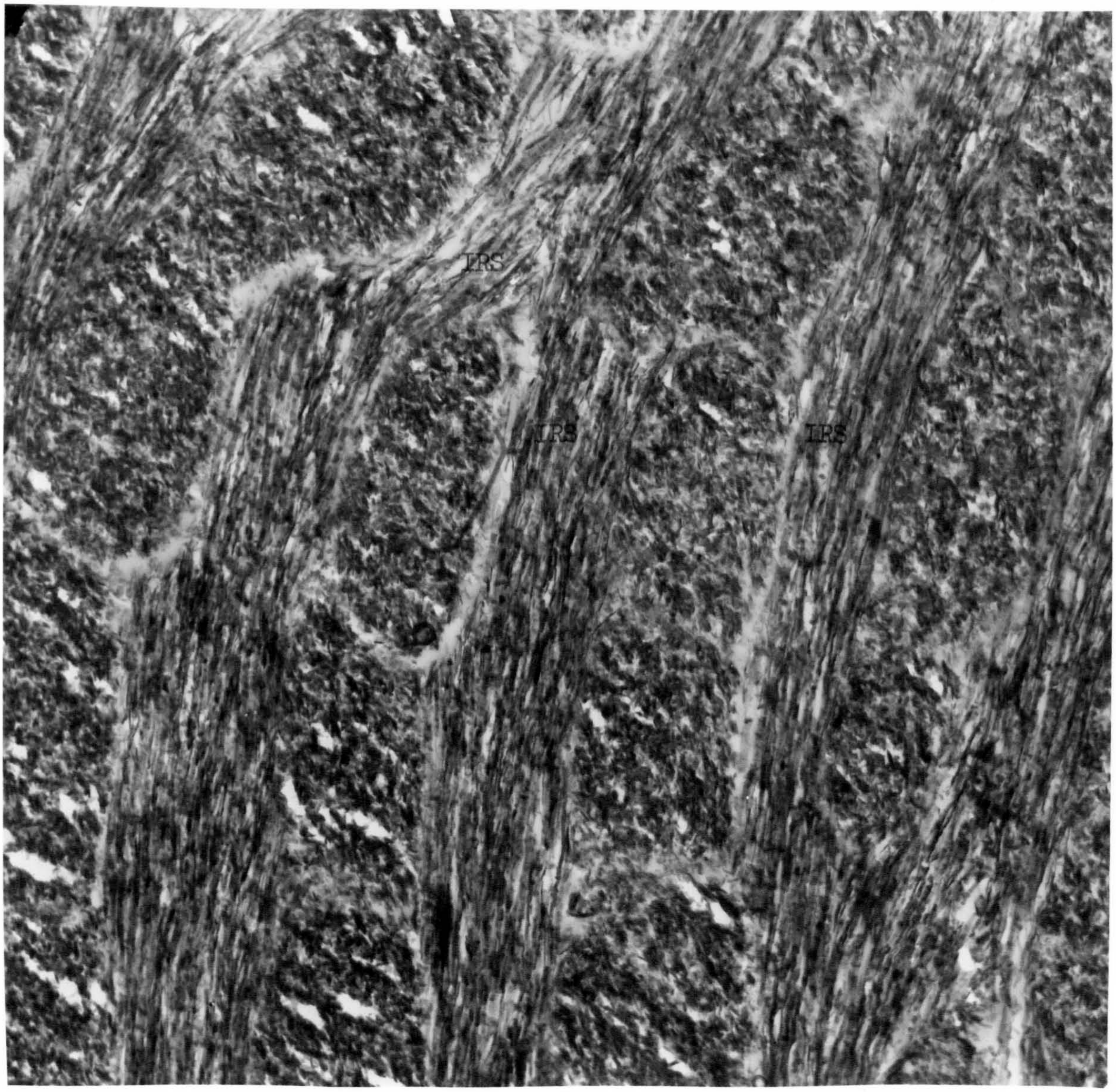


Fig. 2.18A. Sus enamel (X 1050)

Fig. 2.18B.
Sus enamel
(X 1050)

Fig. 2.18C. Bos bovis enamel (X 12600)



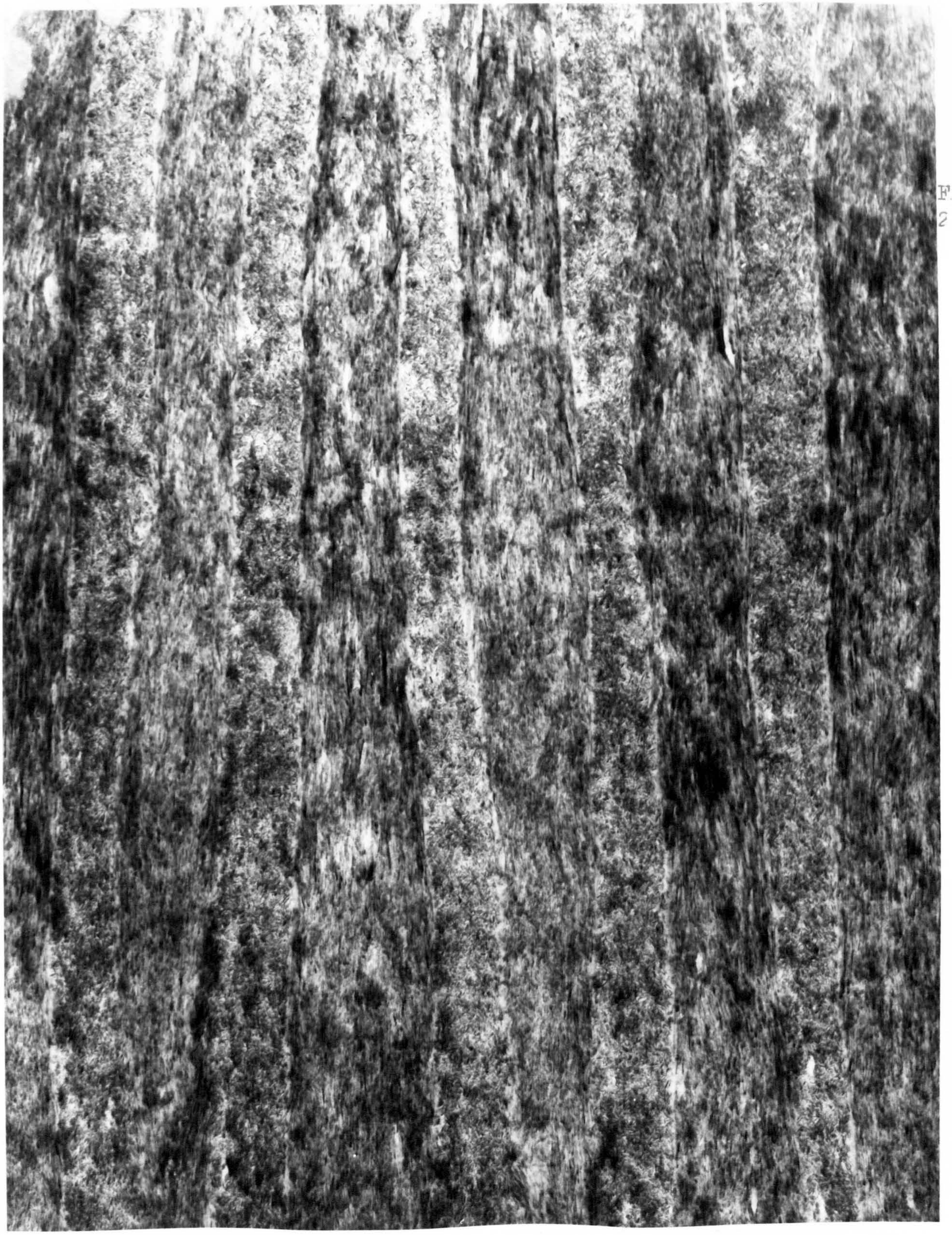


Figure 2.19. Sus: L.S. Prisms (oblique T.S. tooth) (X19900)

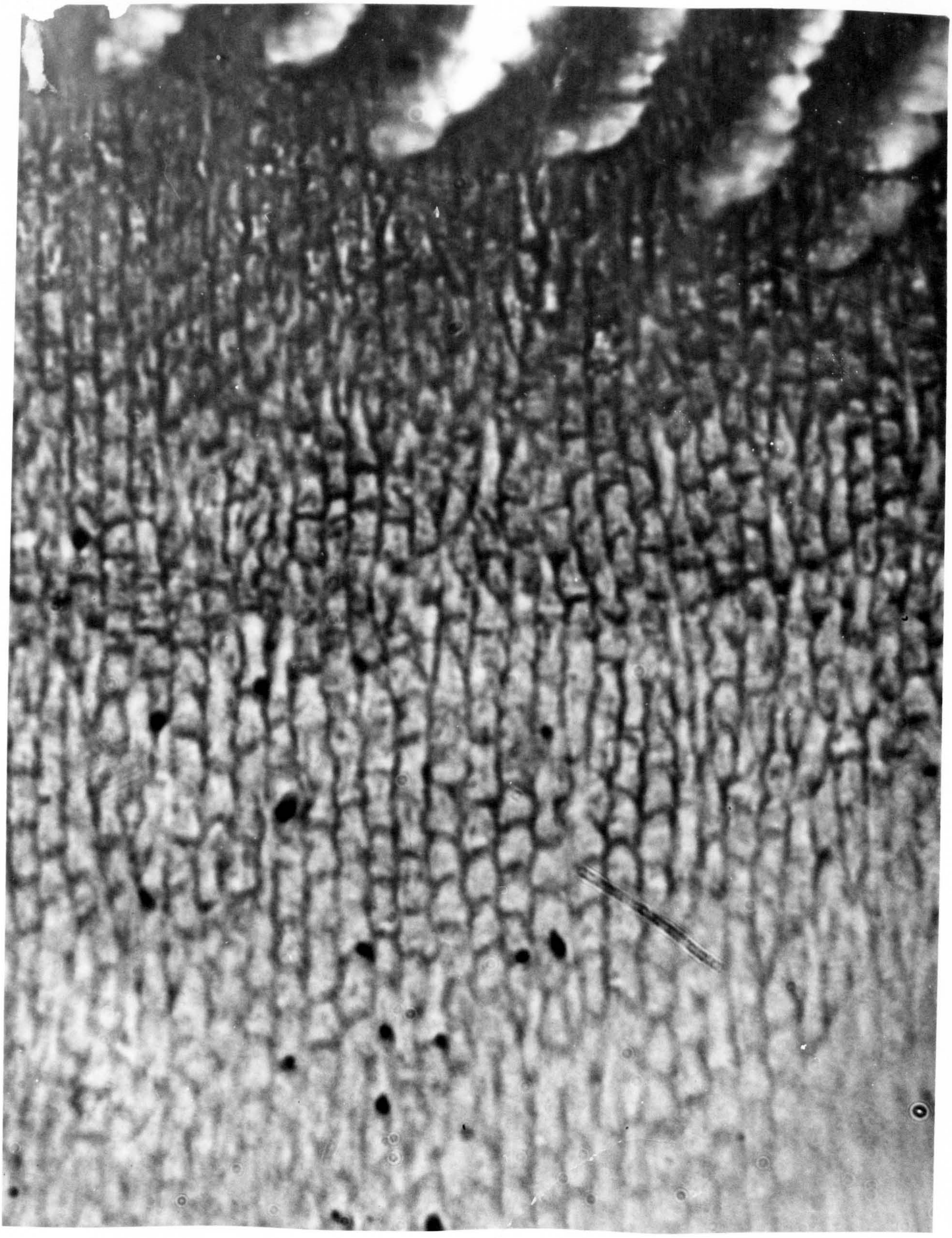


Figure 2.20. Wallabia rufrogrisea: T.S. prisms. (X 1500)

2.4.3. (RESULTS Contd.) SPECIES-, GENUS-, and ORDER-CHARACTERISTIC FEATURES

2.4.3.1: Ungulata - Sus domesticus, Bos Bovis, Equus caballus, Capra hircus. (Pattern 2, Diagram Figure 2.21).

The special feature of the enamel of the Ungulates which made them particularly interesting from the point of view of this study, is the clear definition of the longitudinal rows of prisms which are separated from each other by clearly defined sheets of interprismatic "substance" (Fig. 2.18). There is generally no definable "interprismatic region" between the prisms constituting one longitudinal row. The crystallites are approximately parallel to the long axes of the prisms; diverging to some extent from the centre as visualised in a longitudinal ("picket-fence" plane of) section; and but very little in a transverse ("battlements") plane (Fig 2.19). Thus far the crystallites obey the general rule of being orientated at right angles to the mineralising front. The small divergence of the crystallites from the prism axis in the transverse plane is related to the occurrence of a markedly flat side of the depression in the mineralising front in this case. The crystallites in the plane of the interprismatic sheets are parallel with one another over much larger distances (perpendicular to the long axes of the prisms) than occurs anywhere else in mammalian enamel, except at the true surface and the enamel-dentine-junction. They develop with their long axes perpendicular to the crests of the developing enamel front between the rows of depressions. If the prisms develop at a marked angle to the developing front, as indeed is often the case, then there is a large angle between the crystallites in the prisms and those in the interrow sheets. The longitudinal row arrangement prominent in ungulate enamel was also found in the marsupial species examined and notably in the herbivorous Macropodidae (Macropus- Species not known, and Wallabia rufrogrisea - Fig. 2.20:1) The same pattern (Pattern 2, see Figures 1.2 and 2.13.2) was also found in limited areas in human, monkey, and rabbit enamel; and the pattern found in the enamel of the Rodentia is only modified from this basic pattern by the decussation of the prisms. (See next section 2.4.3.2.) The interrow sheets of parallel-oriented crystallites

Figure 2.21

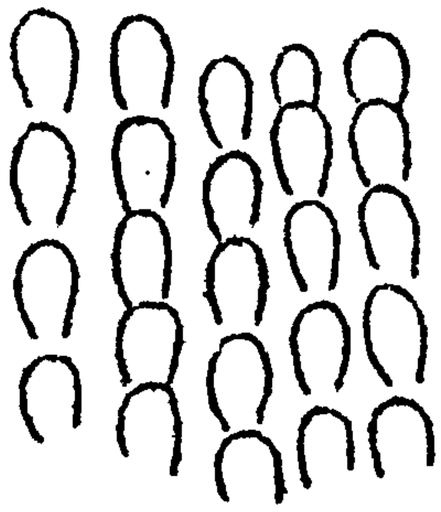


Fig. 2.21.1

Ungulate enamel prisms are sometimes packed together like this, so that there is little correlation between the (vertical) position of the prisms in adjacent longitudinal rows.

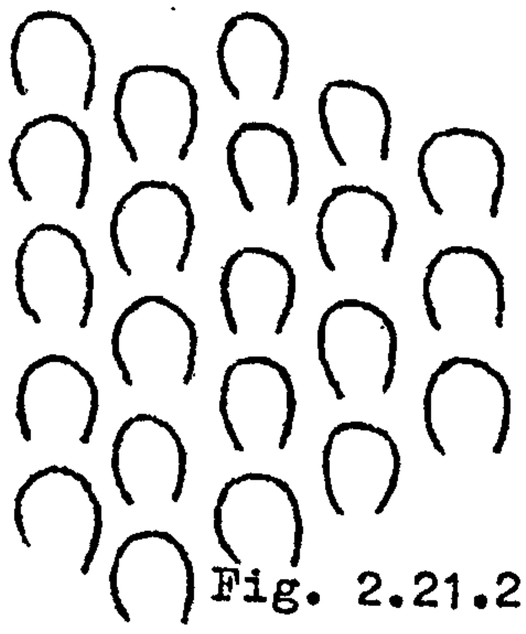


Fig. 2.21.2

It is nearly always possible to visualise the basic hexagonal packing arrangement behind the Pattern 2 arrangement when it is found in human or monkey enamel.

Where Pattern 2 occurs and there are only scanty <u>inter-row</u> regions (e.g. human and monkey enamel) the prisms are "staggered" in alternate adjacent longitudinal rows so that the most prominent parts of the lateral "bulges" of the prisms of one row fit between the prisms in the adjacent rows. Where the <u>inter-row sheets</u> are thicker the prisms are not always at staggered levels in alternate longitudinal rows: a more random arrangement in this respect is often found. (The decussation of alternate transverse rows of prisms in rat incisor inner-enamel determines that every prism in a given transverse row must have the same longitude: - see figure 2.22).

<u>Figure 2.21 .3</u> (opposite). Composite diagram showing the relationship between the shape and position of the depressions in the mineralising front of the developing enamel and the "prisms" in ungulate (Pattern 2) enamels.

It should be noted that the most prominent parts of the honeycombed surface of the developing enamel may be related to regions which will be "within the prisms" (in their cervical parts which are not encompassed by the prism boundary planes and which are in direct continuity with the "interprismatic" inter-row sheet regions) as well as to regions which can be described as interprismatic (i.e. in the inter-row sheets).

Figure 2.21.
Ungulate (Pattern 2) enamel development.

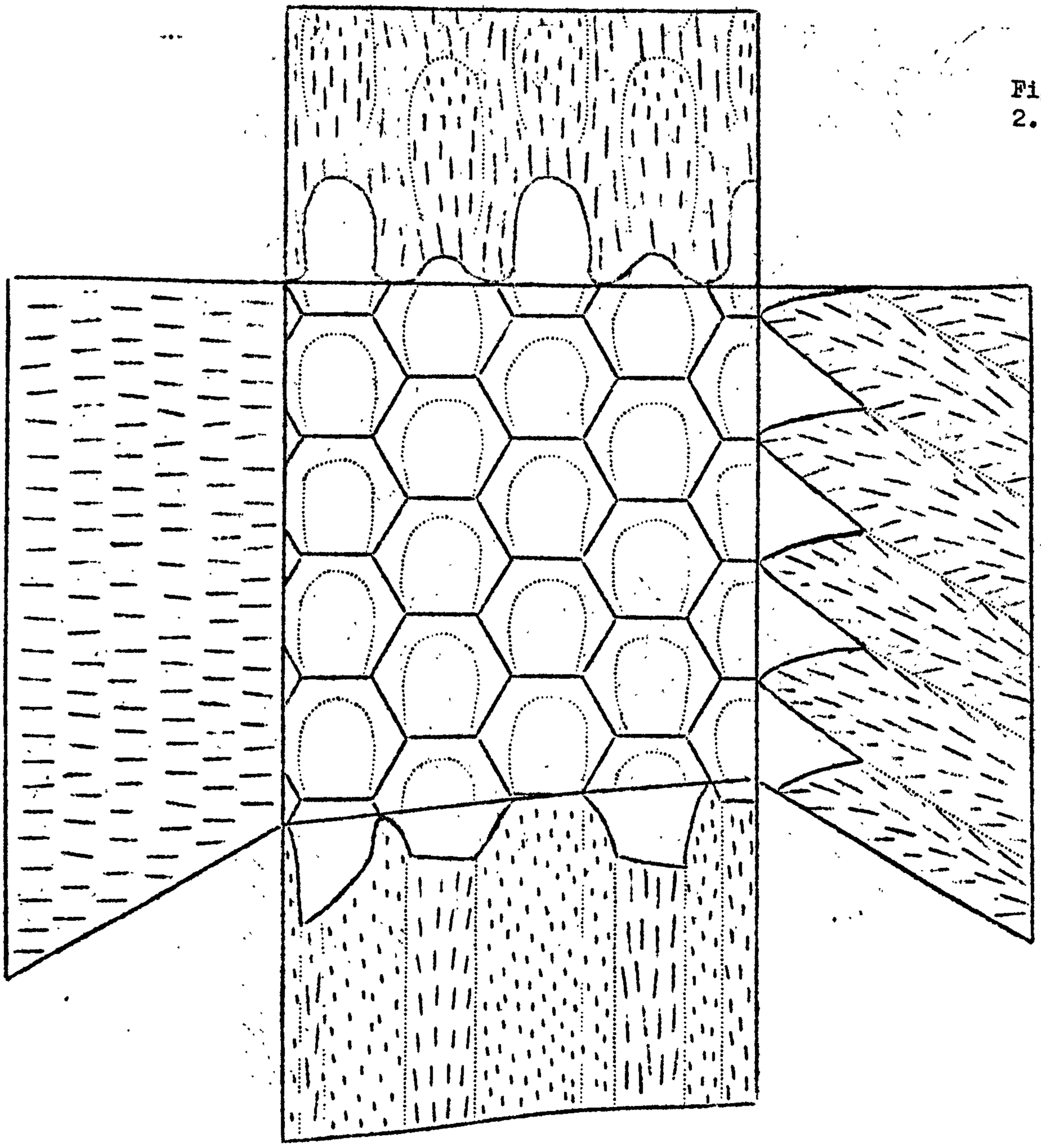
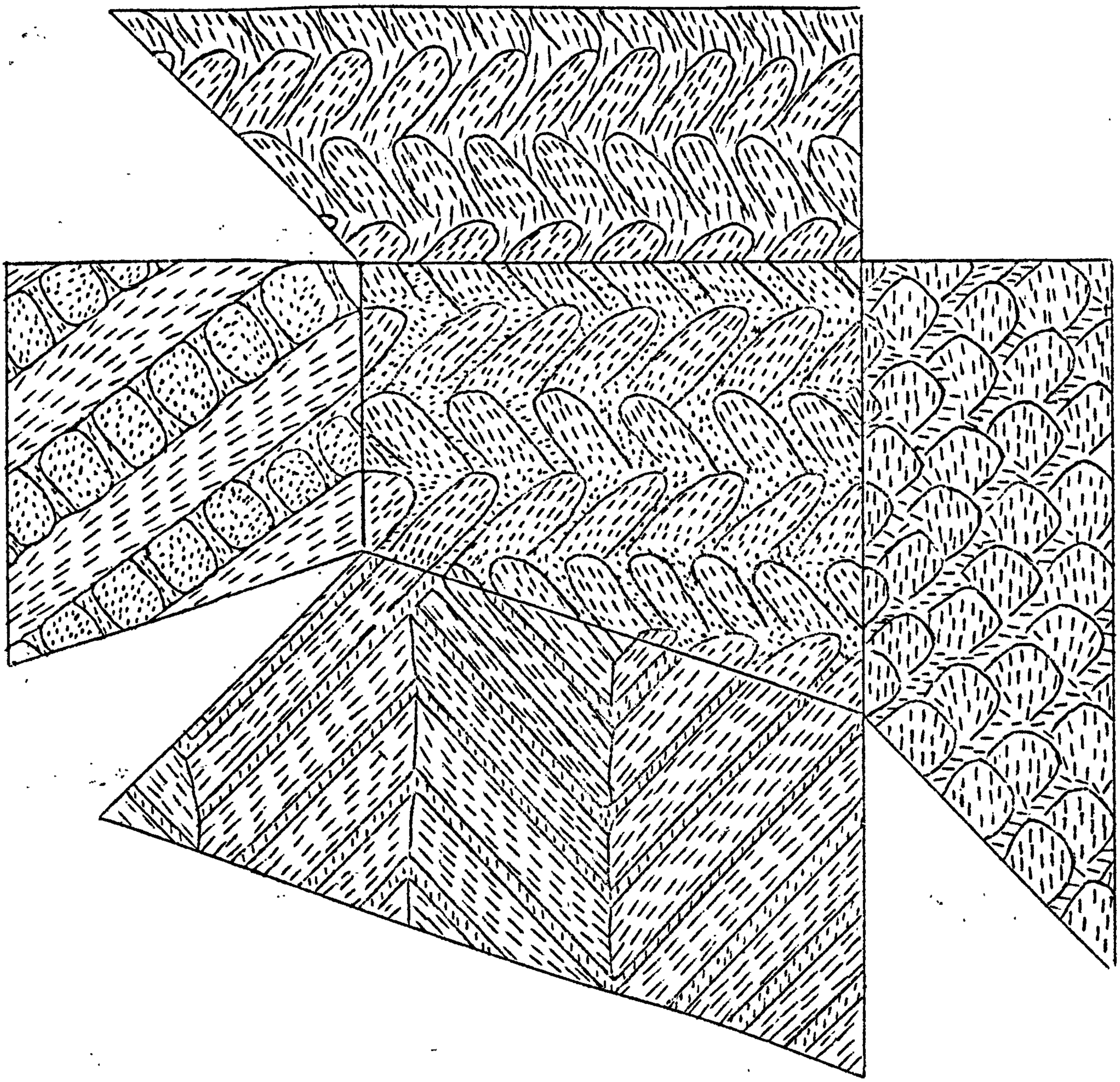


Fig. 2.21.

The hexagonal areas enclosed by the firm lines (on the top surface, i.e. the central area of the diagram) represent the secretory territories of individual ameloblasts. The hexagonal grid represents the outermost parts of the surface of the developing enamel.

The dotted lines represent the sites of the prism boundary planes of abrupt change in crystallite orientation within the enamel, i.e. the line of junction between the intra- and circum-depression crystallites (where the orientation of the c-axes of the crystallites which develop in these two different environments changes), and thus at the same time represent the shape possessed by the depressions in the mineralising front at the last moment before they become completely filled in at each respective dot.

Figure 2.22 (part 1). Diagram illustrating the prism decussation and crystallite orientation patterns in rat incisor inner-enamel.



The diagram shows the outlines of the "prism boundary planes" and (even more approximately) the lengths and orientations of the fragments of sectioned crystallite in the various surfaces of a block of rat incisor inner-enamel. The central area represents a plane of section parallel to the true surface of the enamel: incisal towards the top. The right hand side is a longitudinal section through the tooth and the top area a transverse section. The bottom and left hand areas slope at 45° to the central area, i.e. they are parallel with the long axes of the transverse rows of prisms (every alternate transverse row in the case of the left hand side).

The relationship between the secretory territories of the ameloblasts and the prism boundary planes is shown diagrammatically in the second part of this figure (on the reverse of the following page.

Fig 2.21

are probably equivalent to the "membranes" described by VON EBNER (1906) in "dissected" developing human enamel, and by MUMMERY (1919) in Macropus.

No convincing E/M observations of the development of the HUNTER-SCHREGER bands (Decussation; zone-formation) in Ungulate enamel were made, because the zones are too wide (ca.20 prisms - KAWAI, 1955) and the transition between adjacent zones is too gradual. However, a distinct zoning of the ameloblasts and prisms was noted in the goat material examined by light microscopy.

Our ultrastructural definition of the "prism-sheaths" as lines of junction between fields containing crystallites having a different orientation, leads to the identification of these "structures" within the prisms themselves (SCOTT, 1955). "Prisms within; prisms were noted in pig enamel; i.e. two complete circular prism sheaths (as seen in transverse section), one-inside the other. Spiral prism sheaths; entering the prism from one side and "coiling up" inside; were also seen in pig enamel.

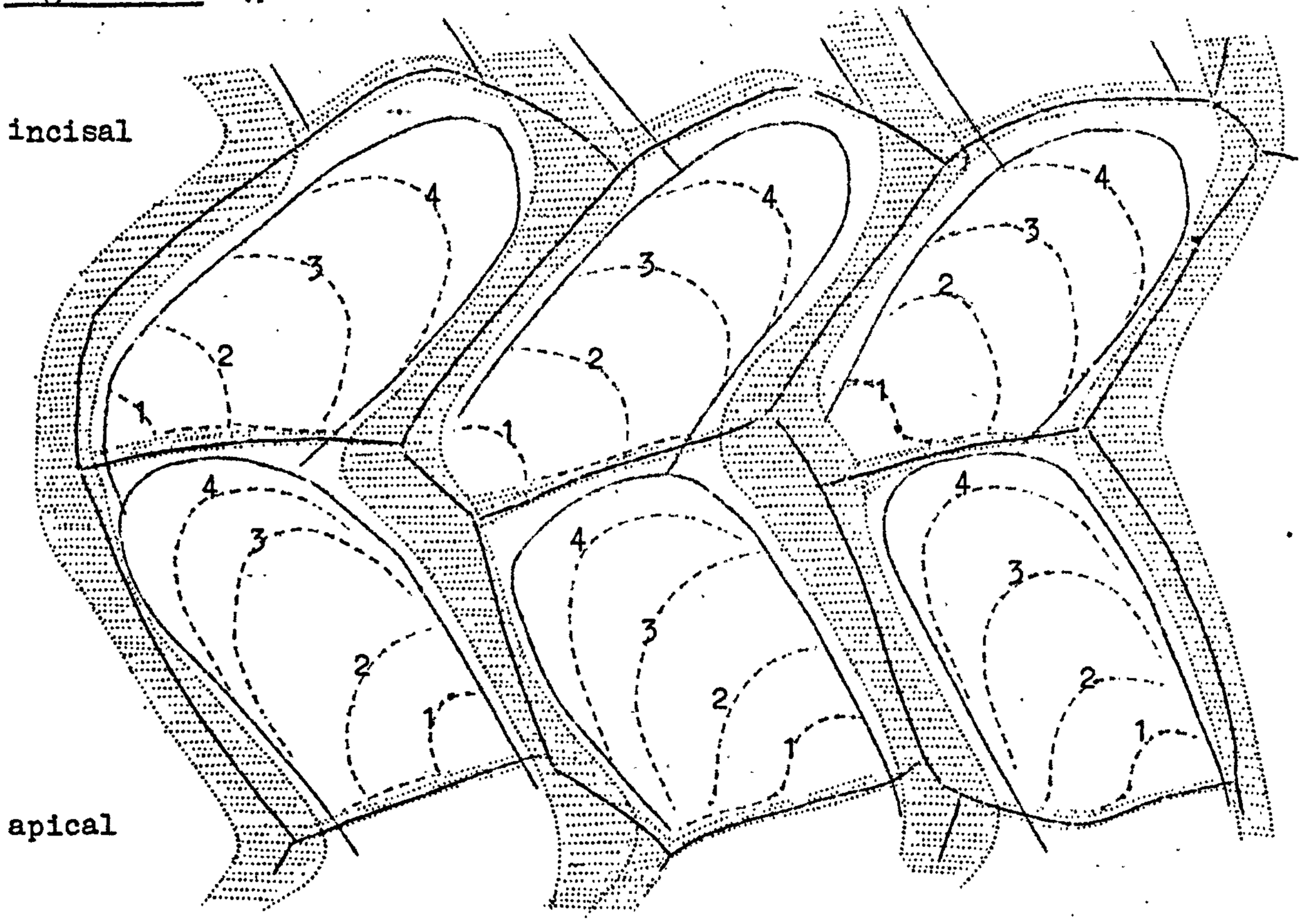
2.4.3.2. Rodent Incisor Inner-Enamel Development.

Rattus norvegicus (Pattern 2B; Diagram Figures, 1.4 and 2.22)

The decussation of the alternate, transverse rows of prisms was fully confirmed. A remarkably clear demonstration of the decussation of the TOMES' process depressions in the developing enamel surface was obtained via the wax reconstructions (Fig. 2.13.1.). Figure 2.17 shows alternate transverse rows of "prisms" filling in from alternate though not exactly opposite sides. The TOMES' processes in this species and in the coypu, are much longer (ca.15μ) than in the other species examined.

The crystallite orientation pattern in rat incisor inner-enamel is a (severe) modification of the ungulate pattern. There is little or no "interprismatic region" between prisms in the longitudinal direction of the tooth. There are well marked longitudinal sheets of interprismatic (parallel oriented) crystallites, but the continuity of these sheets is interrupted by the decussation of the alternate transverse rows of prisms.

Figure 2.22 (part 2). Rat incisor inner-enamel development.



Explanation

The firm lines delineate the secretory territories of individual ameloblasts. The shaded areas show the position which the outermost (most recently formed) enamel occupies: note that thicker areas form first between depressions in the same transverse rows, the thin bridges separating the depressions of adjacent rows form fractionally later (hence there is often a change in orientation between the crystals which form these thin bridges and those which formed first (the shaded areas)). These bridges represent the most apical regions of the "prisms" which form incisally to them. The feint lines represent planes of abrupt change in crystallite orientation within the enamel and thus delineate the position occupied by differently sloping parts of the mineralising front in the developing enamel at the last moment(s) before they abutted against one another. The numbered (1,2,3,4, interrupted, feint, dashed) lines show the sequence of positions occupied by the apical sides of the depressions in the mineralising front during the process of filling in the depressions, i.e. they show the appearance in successive serial sections through the depth of the depressions.

This means that short segments of the longitudinal interprismatic "sheets" must run with their crystallites diverted by the direction of the transverse row of prisms (between the members of which they are situated); that the interprismatic substance of adjacent transverse rows is continuous; and that, therefore, following a line of crystallites along, it would be found to swing gently from one side to the other as it passed between alternately directed rows of prisms. The interprismatic crystallites pass between the alternate rows of prisms in the Myodont enamel type, because they are oriented perpendicular to the surface of the enamel (ignoring the deviation to left or right-in alternate zones), whereas the prisms have a marked incisal inclination.

Myocastor coypus - In the Hystricodont type of enamel, as described by KORVENKONTIO (1934-35), the lamellae of prisms (zones) consist of some five transverse rows. This appears to be quite in keeping with my own findings. However, it is not certain that the prisms adhere rigidly to the zones in which they commence (KORVENKONTIO, 1934-35) and the possibility mentioned by TOMES (1850) that the prisms undulate from side to side in their course from the enamel-dentine junction to the surface cannot be excluded. The truth seems to lie between these two extremes. The junction between the alternate "zones" of decussating prisms is not as distinct as in the rat. The prisms do not cross each other at such a steep angle, and there is often a transitional zone at the junction between decussating zones in which a layer of prisms is parallel with neither zone - alternatively, the prisms at the periphery of one zone may "break away" and join the next zone. This is not the same as the undulation described by TOMES, since he supposed that the prisms are adherent to one lamella. The Hystricodont pattern that I have described differs only in degree from that found and described in the Lagomorpha as the Lagodont type by KORVENKONTIO. In this latter case, however, the transition between adjacent zones or lamellae is really very gradual. The crystallite orientation pattern found in the coypu differs but little from that found in the Ungulata. The width of the zones (diazones, parazones, lamellae) is

Fig. 2.23. Electron micrograph (X 16000) of ultra-thin section of developing coypu rat incisor inner-enamel. The plane of section is parallel to the prisms in one zone (on the left) and cuts those in the next zone (on the right) obliquely. The longest fragments of sectioned crystallite are in the prisms proper in the zone on the left, and in the longitudinal inter-row sheet regions in the zone on the right. The "cells" of TOMES (1850) are the extensive crystal-free spaces at the periphery of the prisms.

bac.
2.2

Fig.

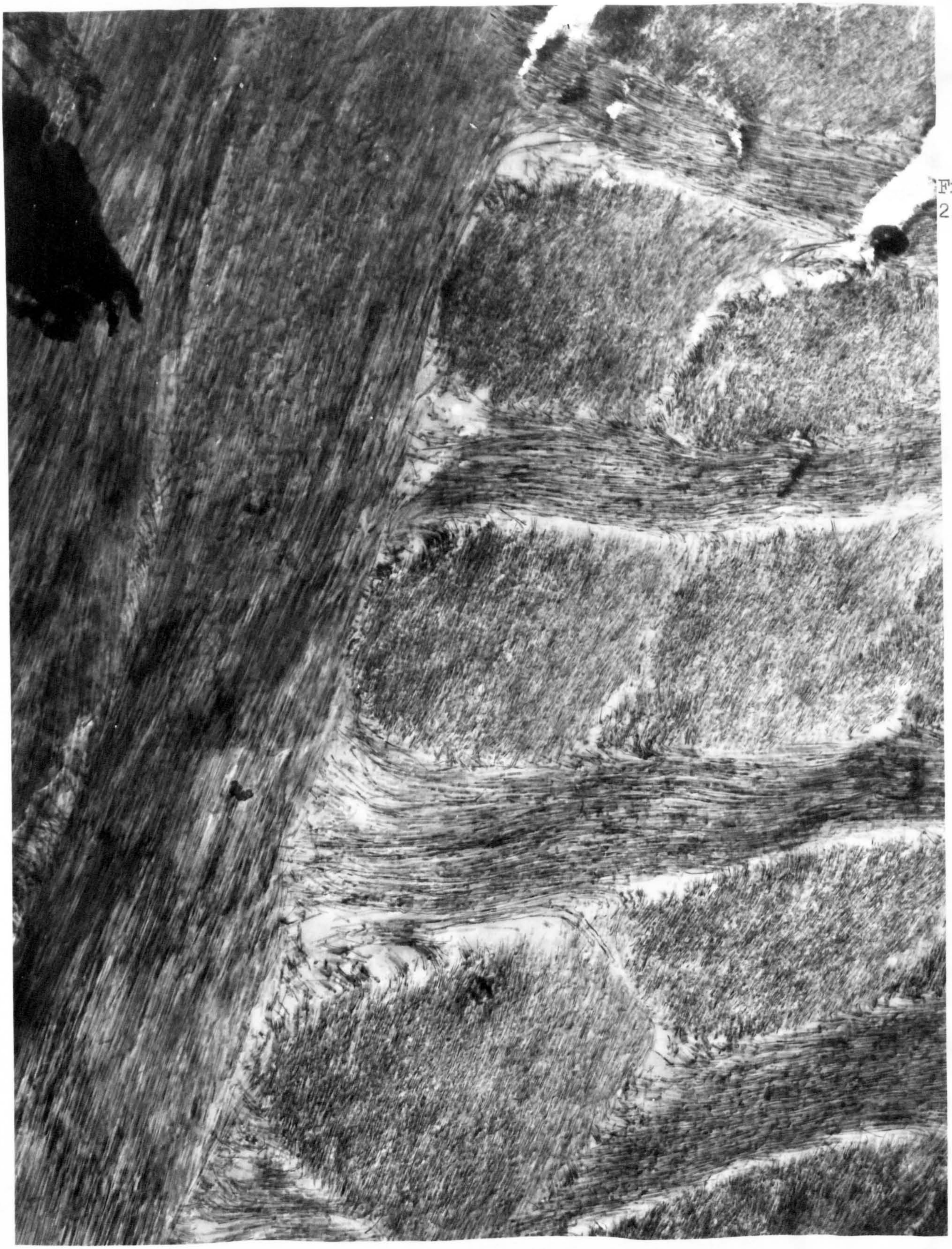


Fig. 2.23.

Figure 2.23. Myocastor coypus: L.S. and oblique T.S. prisms. (X 16000)

sufficient to allow the recognition of well-marked longitudinal interrow sheets (Fig. 2.23) - these again contain mostly parallel crystallites which develop at right angles to the crests of the septae between the longitudinal rows of depressions in the mineralising front. The interprismatic sheets are continuous between the longitudinal rows and at the junction of the transverse rows (zones) : and the orientation of their crystallites is, as in the rat, affected by the change in orientation of the transverse rows (zones).

The "cells" which TOMES (1850) described in the more superficial part of the inner-enamel of the coypu were fairly easily identified after the related problem had been overcome in locating and identifying the tubules in marsupial enamel. These "spaces" occur at the periphery of "prisms"; more often where there is a sudden transition in the direction of the prisms between one zone and the next. (Fig. 2.23). They are considerably wider (ca.1μ) than the tubules in marsupial enamel (ca.0.5μ); are of a limited length (ca.5μ); and are connected neither with the dentinal tubules nor the true surface of the enamel. The only contents which I could identify were occasional crystallites and the methacrylate embedding medium.

KIONOBLASTS (SAUNDERS, NUCKOLLS and FRISBIE, 1942; SYMONS, 1955) were identified amonst the coypu incisor ameloblasts. They were recognised first in $\frac{1}{2}\mu$ sections stained with crystal violet and basic fuchsin. (Fig. 2.24.1.) and later in ultra-thin sections of the same blocks in the electron microscope (Fig. 2.24.2). One can only agree with the opinion of KEREBEL and GRIMBERT (1958, 1961) that they are degenerating cells.

Fig. 2.24.1 Photomicrograph (X 2500)

& 2 Electron micrograph (X 24000) of transverse section of coypu incisor ameloblasts at the level of their nuclei. Two (very electron dense) cells show extensive degenerative changes which cannot be attributed to the quality of the fixation (compare these two cells with their neighbours). The same cells were first identified in the light microscope image of adjacent sections stained with crystal violet and basic fuchsin: they are held to be "kionoblasts" (SAUNDERS, NUCHOLLS and FRISBIE, 1942).

Fig. 2.24.1. is a photomicrograph of a $\frac{1}{2}\mu$ thick section of the same methacrylate embedded block from which the ultra-thin section (Fig. 2.24.2) was prepared.

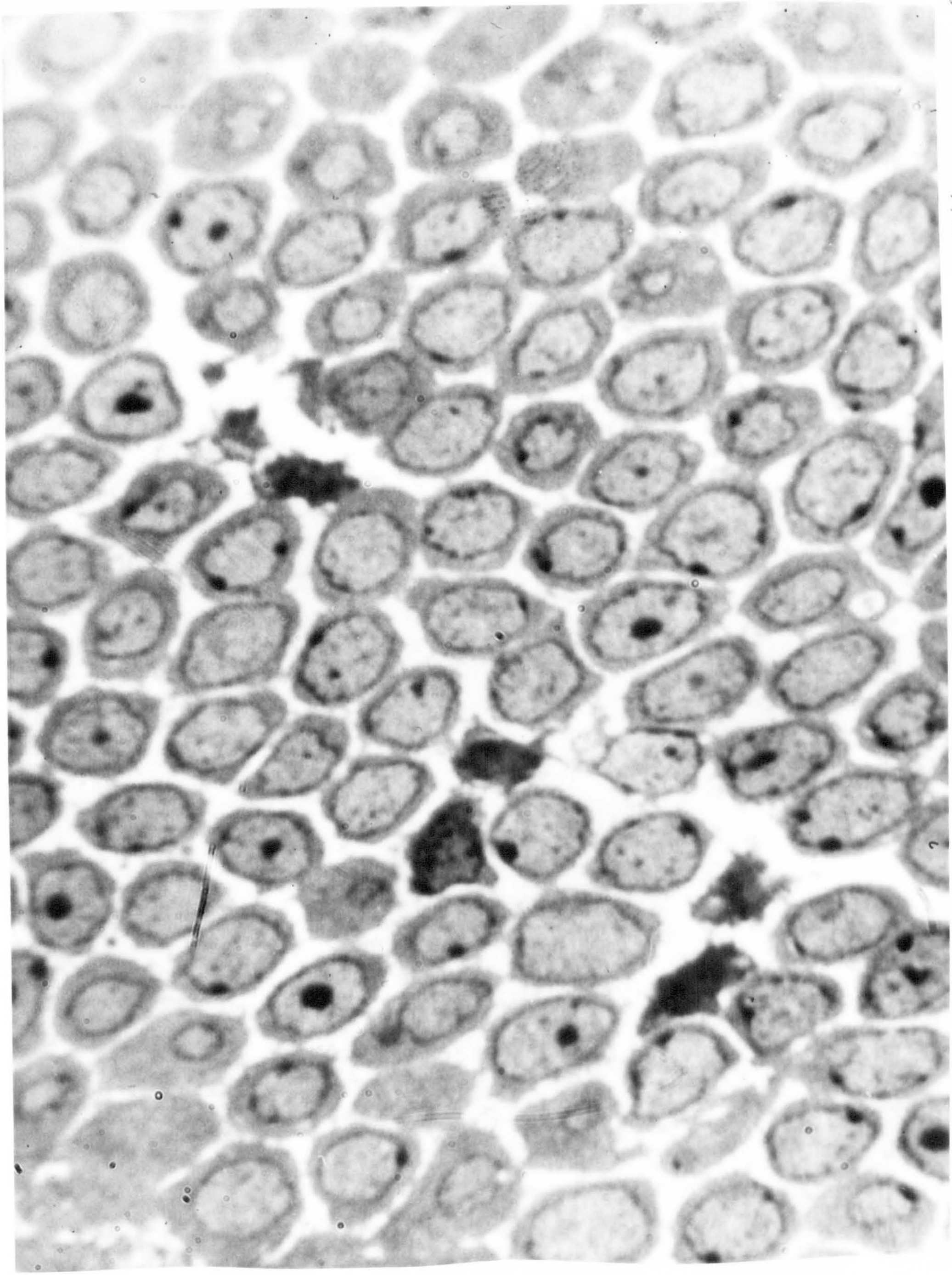


Fig. 2.24.4

Figure 2.24.1. Myocastor coypus: T.S. ameloblasts and "kionoblasts". (X 2500).

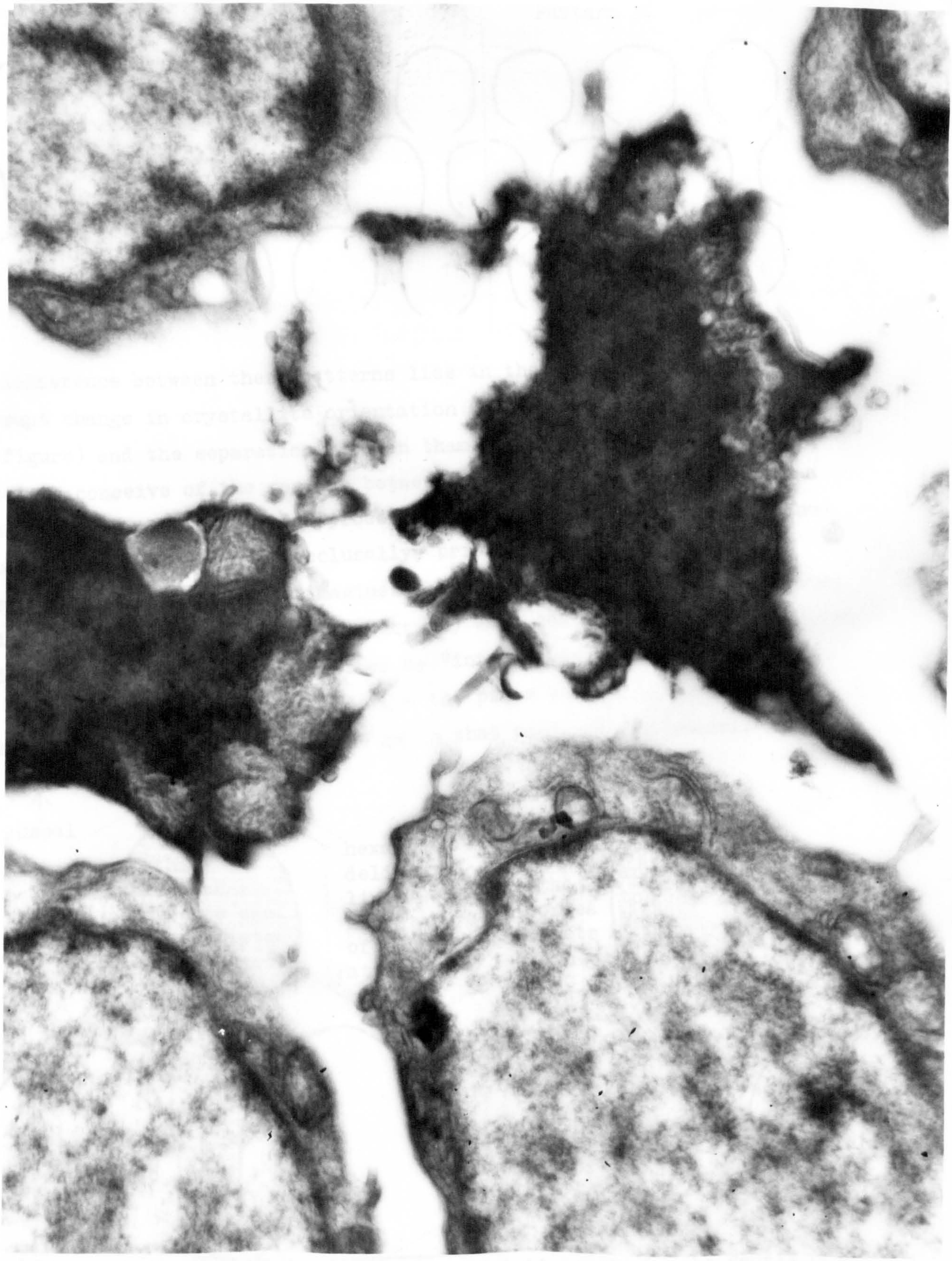


Figure 2.24.2. Myocastor coypus: "kionoblasts" (X 24000)

2.4.3.3. PROBOSCIDEA: Loxodonta africanus(PATTERN 3 -Diagram figure 2.25.)

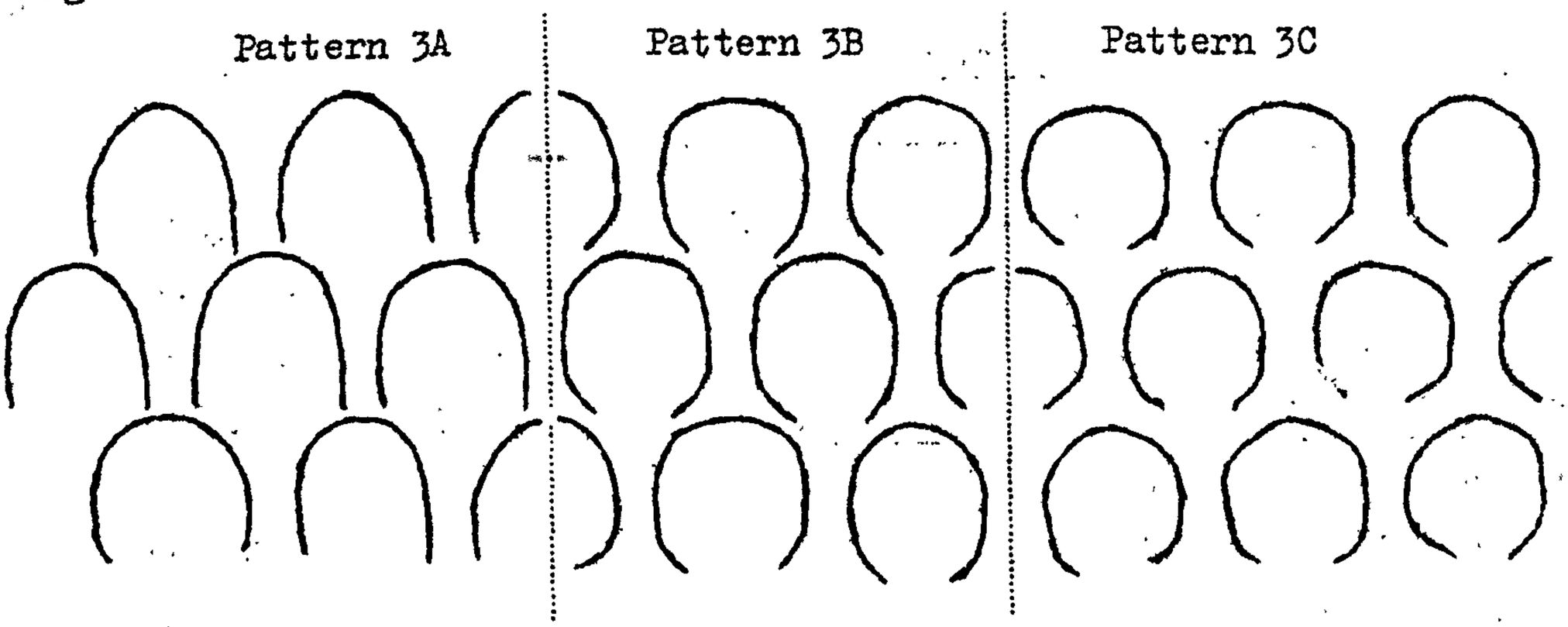
A high incidence of PATTERN 3 was found in this species and this, combined with the articular extent of the prism boundaries may be held responsible for the occurrence of that remarkable close-packing arrangement of the prisms which led VON EBNER (1906) to christen the "Flügelfortsätze" - the "winged processes" of MUMMERY (1916, 1919) or the "alar processes" of CHASE (1927 A). The "winged process" of these authors is the continuation of the prism-substance (i.e. uninterrupted by any sudden change in crystallite orientation) of the cervical side of one prism between the "gap" between the two prisms adjacent to it on its cervical side (Fig. 2.25).

There are no regions which can properly be defined as interprismatic in PATTERN 3A (Diagram figure 2.25). The winged process region contains the crystallites with the maximum deviation from the prism axis; and these regions are equivalent in this respect (and also in respect of their developmental position and in that they are "interprismatic" from the point of view of the prisms in the adjacent (more cervical) transverse row) to the interrow sheet regions in Pattern 2 and the true interprismatic regions in Pattern 1.

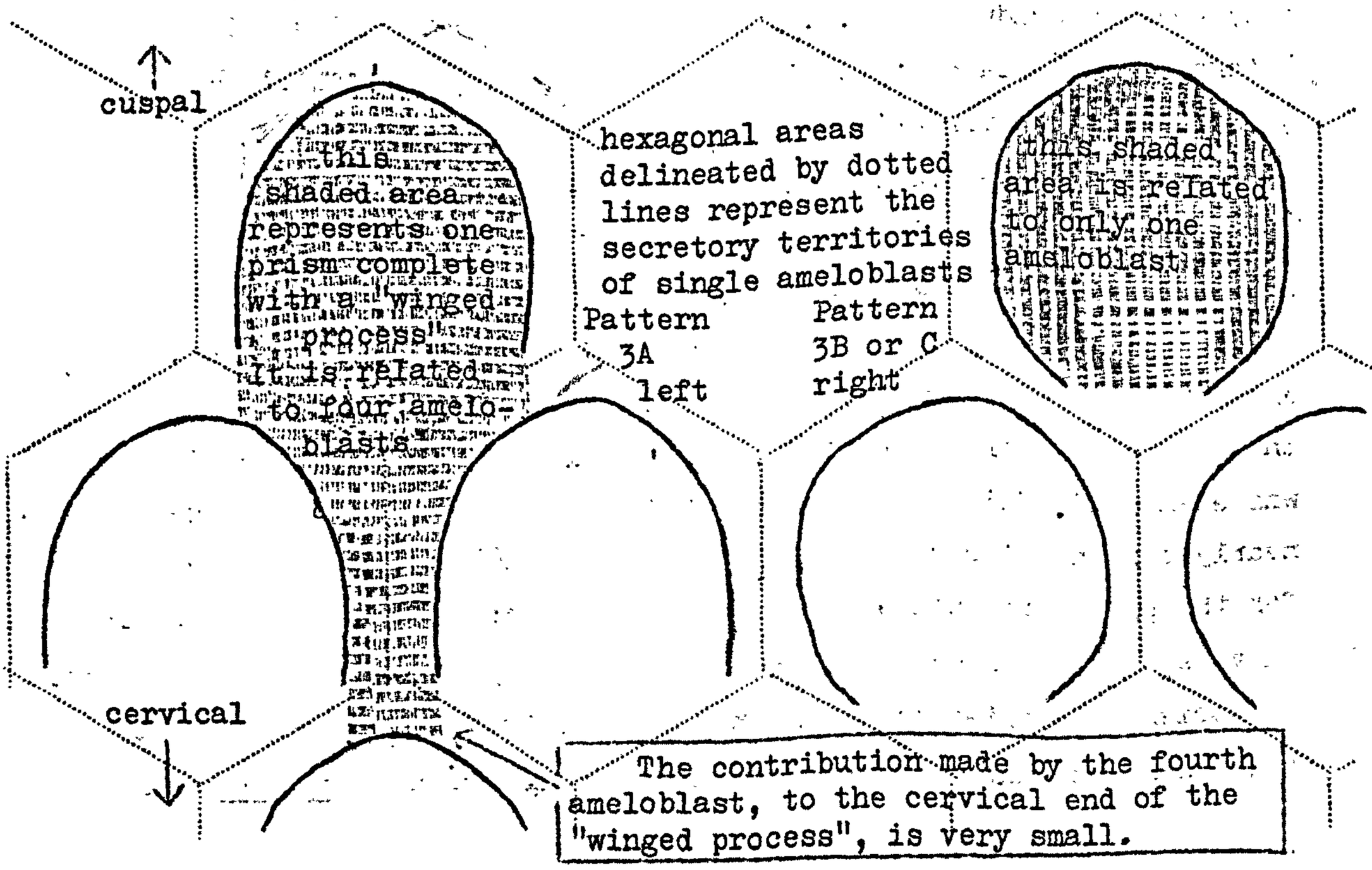
The width of the domains in which crystallite orientation only changes gradually (i.e. the prisms in PATTERN 3A) in the "Picket fence" plane of section may vary greatly in PATTERN 3A according to the variation of the plane of section from the true longitudinal plane (see figure 2.11). The (longitudinal)width of elephant enamel prisms through the axis of their "winged processes" can be very large (ca. 12μ).

Elephant enamel was found to be substantially harder, closer to the ameloblasts, than in any other species (excepting the case of the true surface enamel in those species in which it was examined). This made it very difficult to study in other than nearly tangential sections. A tendency was noticed in these sections for the prisms to separate one from the other: this is not surprising in view of the absence of a binding continuity between adjacent prisms. The prism boundaries extend so far cervically towards their neighbours that there is only the narrowest neck through which a gradual change

Figure 2.25



The difference between these patterns lies in the extent of the planes of abrupt change in crystallite orientation (represented by the lines in this figure) and the separation between them. In Pattern 3A it seems logical to conceive of the regions between adjacent prism boundaries in a horizontal direction as "winged processes" belonging to the region circumscribed by the neighbouring (occlusally) prism boundary. However, in Pattern 3C one more naturally imagines the prism boundaries to be complete, (cylinders, i,e. circular in cross-section) when the remaining regions outside "the prisms" may be described as "interprismatic". More important than such verbal argument is to see how the prism boundaries are related to the secretory territories of the cells that produced the enamel.



The contribution made by the fourth ameloblast, to the cervical end of the "winged process", is very small.

in crystallite orientation can be traced joining them together This observation could suggest the reason for the ease with which VON EBNER and MUMMERY managed to tear apart the prisms in developin enamel of this particular pattern.

2.4.3.4. PRIMATES: Homo sapiens and Rhesus macacus.
(PATTERNS 1, 2 and 3; PATTERN 3, Fig. 2.25)

All the three basic prism PATTERNS may be found in human and monkey enamel. PATTERN 1 is common in the enamel over the tips of cusps and incisal edges. In human "lateral enamel" (POOLE and BROOKS. 1961) PATTERN 3 is found more commonly than PATTERN 2, and where t latter is found it is not associated with the thick interrow sheet regions found in the Ungulata and Macropodidae. The PATTERN 3 arrangement (PATTERN 3B;Appendix figure 2.35.) differs from that found in the elephant (Fig. 2.25.) in that the domain boundary planes (prism sheaths) often extend through more than half a circle as seen in transverse section. In other words there is a transition towards PATTERN 1 and although "prism" and "interprismatic" regions are still continuous with each other, one feels intuitively drawn towards talking of interprismatic regions, rather than "winged processes". However, the interpretation of the crystallite orientation (domain) pattern is rather more important than the words used to describe it. The occurrence of more-complete prism boundaries (transverse sectional shape) indicates that each prism is only related to one amel:blast in patterns 3B and 3C. The transition is very gradual between these patterns. It is only because we can conceive of all the crystallites belonging to "prism" domains in PATTERN 3A that we can say that each ameloblast is related to 3 prisms. In fact, the portions which an ameloblast contributes to the two prisms situated on its cuspal side in PATTERN 3A are exactly equivalent to the regions which one can begin to define as inter-prismatic in situation in PATTERNS 3B and 3C. Tbe differences betwe these patterns lie not in the relative distribution of material on different sides during the filling-in of the depressions, but in the extent of the plane of abrupt change in orientation (prism sheath) between the intra- and circum-depression crystallites.

Fig. 2.26. Electron micrograph (X 12850) of near-tangential section of the surface of developing cat deciduous premolar enamel, showing the depressions in the mineralising front filling in predominantly from one side.

The section passes through the junction between two (HUNTER-SCHREGER band) zones: the depressions in the lower (more cervical) half of the field are filling in from SSW to NNE, whereas they have filled in from ESE to WNW in the upper part of the field.

Fig. 2.27. Low power electron micrograph (X 4200) of near-tangential section of the surface of developing (Puerto Rican) manatee enamel, showing the depressions in the mineralising front filling in.

A number of circular (in cross section, i.e. Pattern 1) prism boundaries can be seen. Otherwise the prisms fit the description of Pattern 3C (Fig. 2.25.).

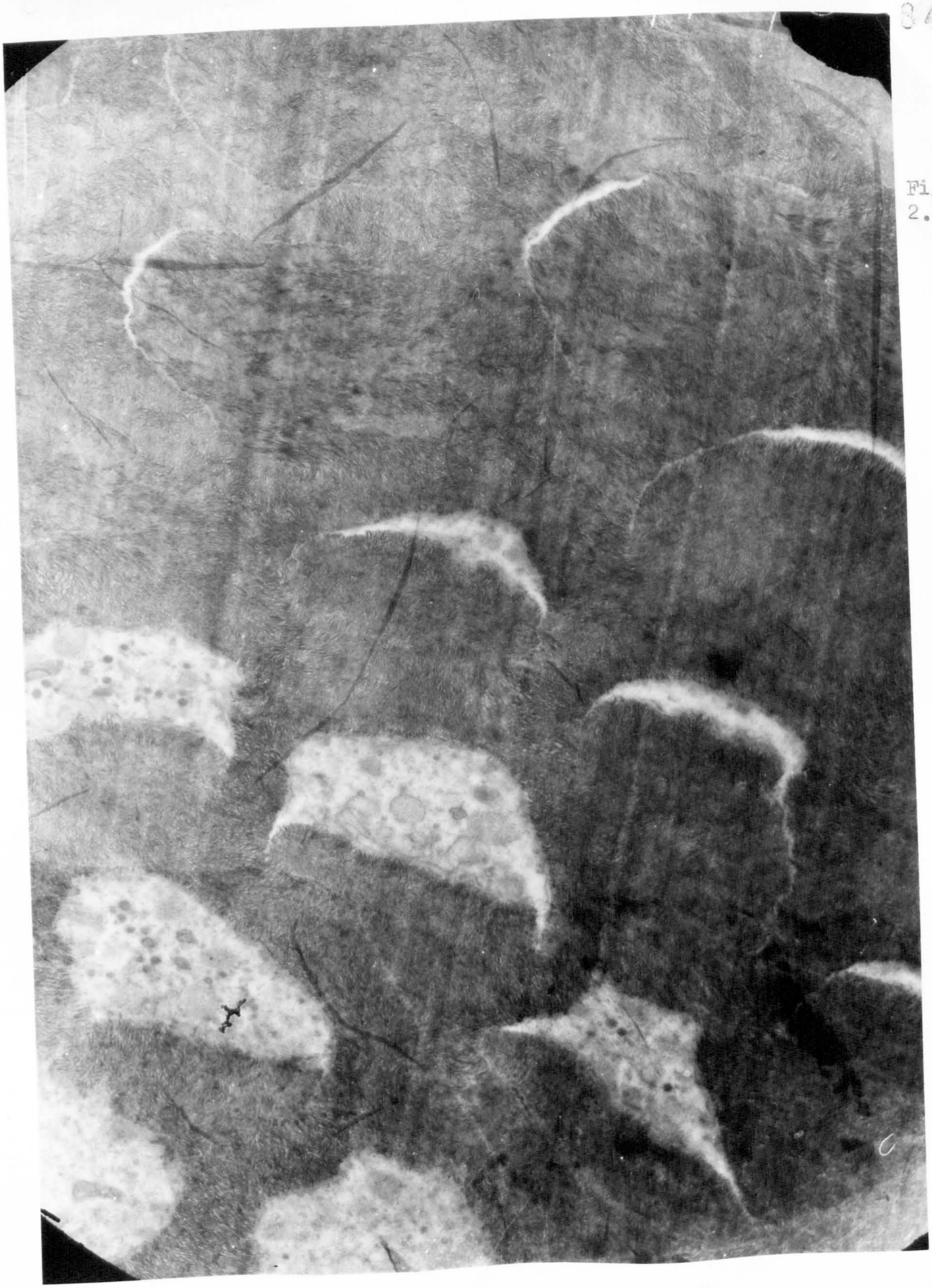


Fig. 2.26

Figure 2.26. Felis: prisms filling in. (X 12850)

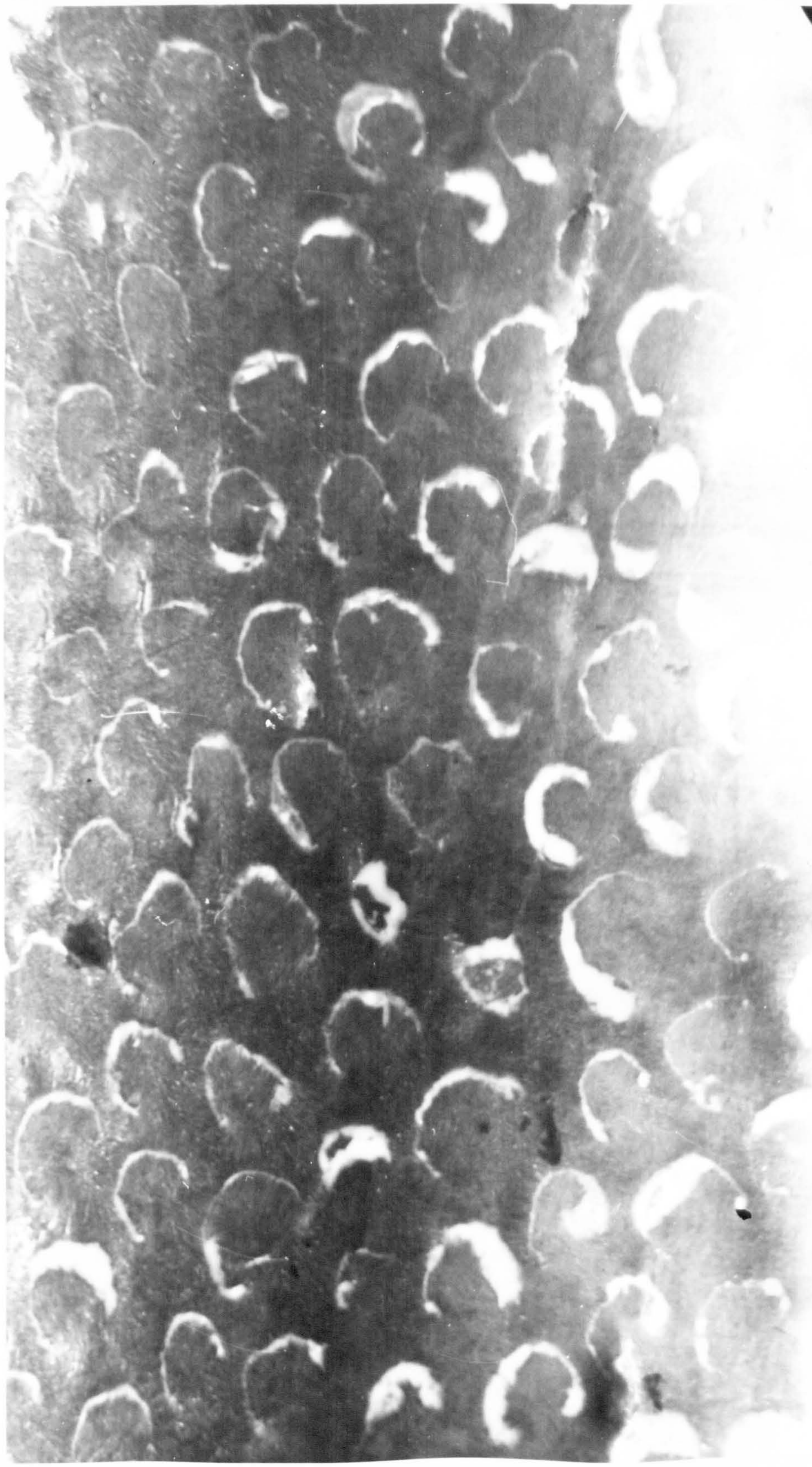


Fig. 2.27.

Figure 2.27. Trichesus latirostris: prisms filling in. (X 4200)

.26. 2.4.3.5. CARNIVORA: Felis domesticus (Pattern 3B or 3C, Figure 2.25)

Cat enamel was found to possess prisms having a cross-sectional shape shown diagrammatically as Pattern 3B or 3C in fig.2.25. The most marked difference from human and monkey enamel lies in the width of the decussating zones. The HUNTER - SCHREGER band-zones in cat enamel are only some five to eight prisms wide and the demarcation between adjacent zones is very sharp. It was possible to study the mode of development of the zones (in honeycomb plane sections of the developing front) in electron-microscopic fields as small as that available at a magnification of 5000 times. The depressions in the mineralising front of the developing enamel related to the prisms in alternate zones fill in from alternate sides: even from nearly opposite sides in some cases in the formation of cat enamel. This feature could also be seen in the developing human and monkey enamels (but only within one electron-microscopic field at much lower magnifications, e.g. at 1000 times).

2.4.3.6. SIRENIA: Trichecus latirostris (Patterns 1 or 3C, Figure 2.13.1,2.27 and Appendix figure 2.39.)

The manatee possesses Pattern 1 enamel, which differs from the carnivore and primate types in the presence of a much greater proportion of "interprismatic regions" and in having complete(circular in cross-section) "prism sheaths".

The absence of decussation of the prisms seems to be associated with the fact that all the surfaces of the depressions in the mineralising front of the developing enamel "grow" at more or less equal rates: i.e. there is no preference for a side from which the filling in of the depressions will occur, as is observed in the development of decussating prisms in both Pattern 2 and 3 enamels.

There is no general correlation between the principal planes of section and the profile (i.e. picket fence or battlements) of the developing enamel surface. In fact a sort of "battlements" appearance is the one most commonly found in any section cutting the prisms longitudinally.

Further comparative anatomical observations of the cross-sectional outline of enamel prism boundaries (made by light microscopical methods on adult, i.e. fully formed mammalian enamels) are considered in Section 5.3.

Figure 2.28.

Photomicrographs of Haematoxylin and Eosin stained 8 μ sections of a decalcified, developing Bennett's wallaby incisor.

Fig. 2.28.1. Enamel.

The field covers the region at the inferior angle of this tooth in which the prisms decussate. The very dark lines are the "fibres" of TOMES, i.e. those parts of the enamel "tubules" which remain after acid decalcification. The "tubules" follow the course of the enamel prisms and cross over each other where these do likewise.

Fig. 2.28.2. Enamel, ameloblasts, other enamel organ cells and dilated capillaries.

Enamel formation is virtually complete at the level photographed. The enamel tubules are not seen as close as this to the ameloblasts during active enamel production, when there is usually a thick layer of intensely basophilic matrix between the ameloblasts and the region in which the "tubules " can be differentiated.

The exaggerated spiral course of the "tubules" in this figure is probably due to excessive shrinkage of the "enamel matrix" at some stage during the decalcification and embedding procedures. The somewhat different nature of the organic remnants of the "tubules" renders them less subject to the shrinkage suffered by the rest of the decalcified organic matrix, and this results in a spiralling of the tubular contents. The amplitude of this spiral does not exceed the prism repeat distance (i.e. the "diameter of the prisms").

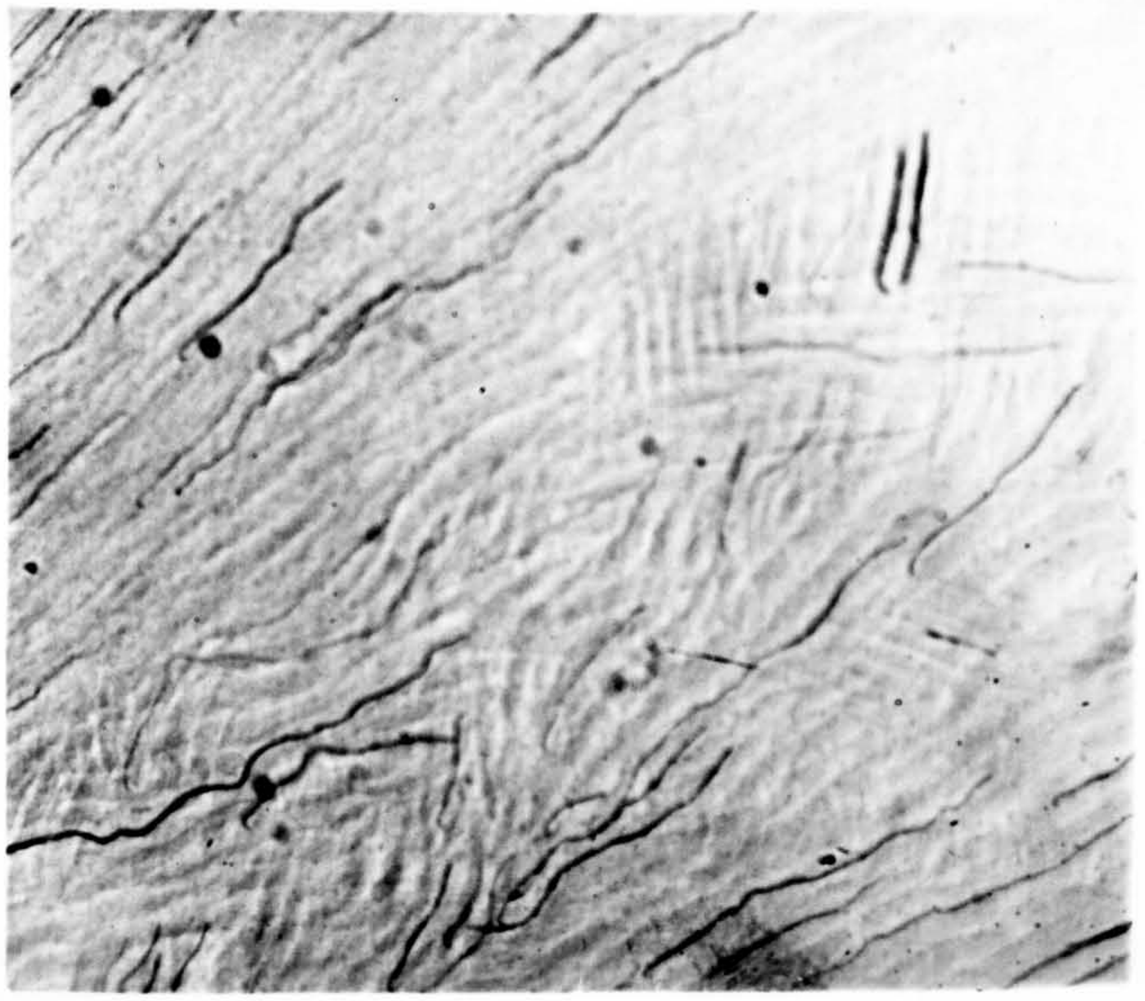


Fig. 2.28.

Fig. 2.28.1. Wallabia rufrogrisea. Enamel of lower incisor showing decussation of prisms and "tubules". (X 450).

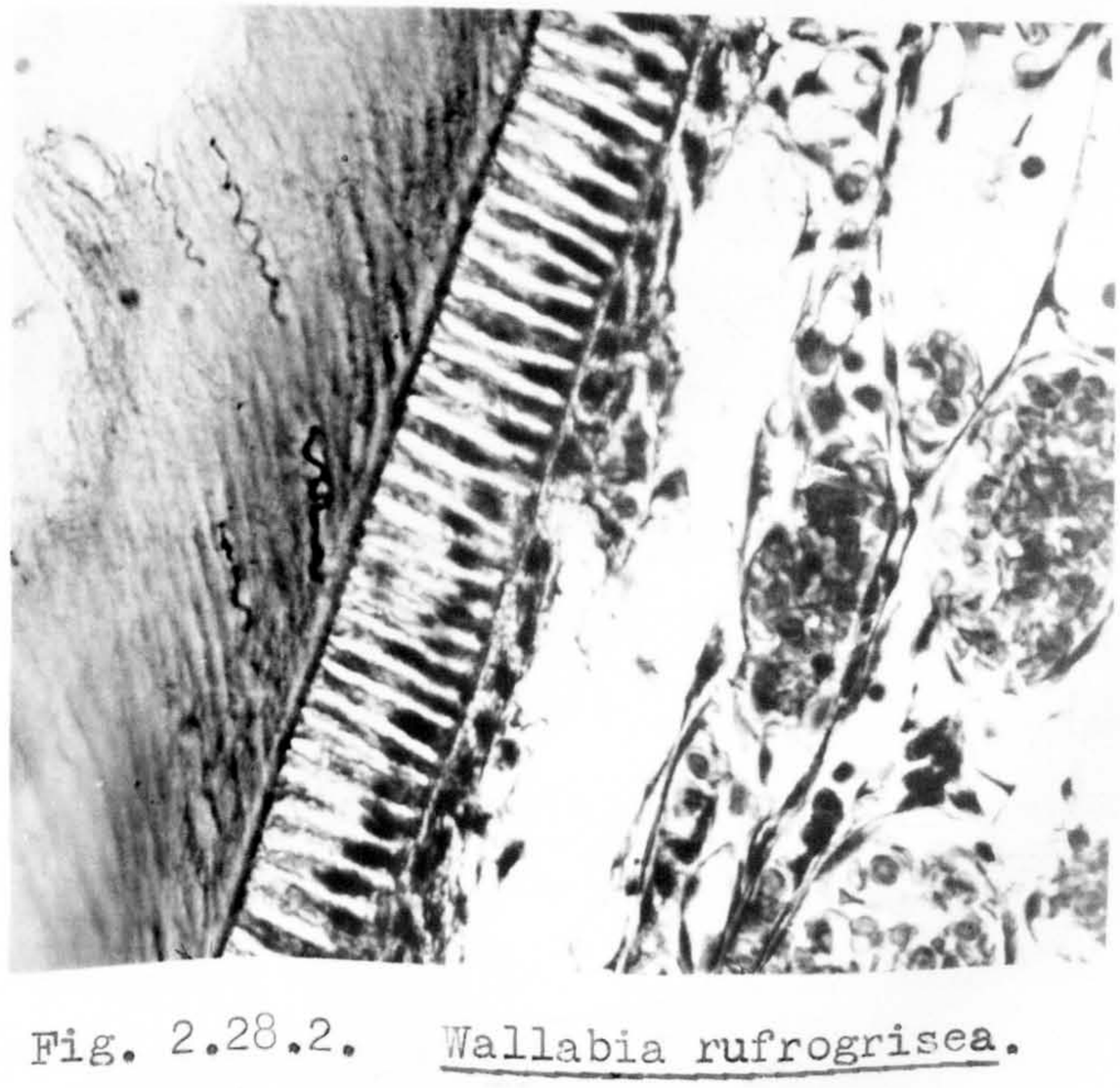


Fig. 2.28.2. Wallabia rufrogrisea. Enamel organ, ameloblasts and enamel with spiral enamel "tubules". (X 450).

2.27 2.4.3.7. MARSUPIALIA: Trichosurus vulpecula, Didelphis virginiana, Didelphis nudicaudata, Wallabia rufrogrisea, Macropus (? species), Pseudocheirus convolutor.

(PATTERN 2; Figures 2.12.2., 2.13.2., 2.15.2., 2.20., 2.21.2.)

Marsupial enamel bears a close resemblance to the Ungulate pattern, with easily defined, longitudinal rows of prisms, and longitudinal sheets of interprismatic substance between these rows. My main interest centred on the identification of the so-called tubules. A lot of trouble was experienced in establishing reliable criteria for their identification, since they are seen (in ultra-thin sections) as defects in the generally even distribution of crystallites in the developing enamel and are difficult to distinguish from artefact tears in the sections. It had been my practice to"scan" my sections in the electron microscope at magnification of 5,000 x and this was probably responsible for the delay in picking up the"tubules." At a magnification of only 1,000 x it was possible to see the equivalent regions in adjacent serial sections; and it was then possible to determine that the pattern of defects - whose identification as tubules was dubious - was identical. The presence of methacrylate embedding medium in these defects was regarded as further confirmation of their "tubular" nature.

It was not possible to resolve any structure in the (presumably) organic component of the tubules, and in this way it resembles the rest of the organic matrix between the enamel crystallites and in the "prism-sheath" regions. The only contents of the "tubules" were occasional isolated crystallites which passed through or across them.

The tubules seem to be more commonly situated within the prisms. I could not determine that their location within the prisms followed any particular rule, and they could be found adjacent to the prism-sheath or within the centre of the prism. I am not certain that the tubules never develop in the interprismatic sheets.

Light microscopic observations: The enamel tubules were preserved as basophilic "fibres" in both the developing and mature, decalcified enamel in all the marsupial species examined. In the mature tissue they were the only elements to be retained and their relationship to the original prism architecture could not be ascertained. In less

2.28

"mature" decalcified enamel they sometimes showed a marked spiral configuration (Fig. 2.28 - This was probably due to shrinkage in the tissue during and after decalcification), but they were always well differentiated from the pale staining (acidophilic) "matrix." In young enamel the basophilic "fibres" were obscured in the intense basophilia of the whole tissue. They could not be differentiated in the young enamel zone even in the trichrome stained material. It was not possible therefore, to determine the relationships of the "tubules" ("fibres") to the honeycomb of the developing enamel. I believe that the tubules (i.e. the differentiated organic content of them that resists decalcification and is retained as the "fibres") are not differentiated in the young enamel zone, and that it is only in "maturing" enamel that they first appear (see Discussion Chapter 9). (The PATTERN 2 arrangement of the prisms was found in all the marsupial species examined. It was described and figured by SHOBUSAWA (1952) and figured (though not described) by MUMMERY (1916), HÄUSELE (1932) and MARCUS (1931)).

2.4..4 Commencement of Amelogenesis (Figure 2.29).

The electron microscopic evidence confirms the well-established view that enamel deposition awaits the formation of dentine and that a part of the dentinal matrix (pre-dentine) is calcified (as evidenced by its striking increase in electron density) before enamel deposition commences. The mineralisation of the first formed dentine appears to proceed with the same random order that is found in the later stages of its growth; i.e. the mineralisation does not progress as a continuous front beginning at the enamel-dentine junction (in this it differs from enamel), but rather it progresses outwards from a number of centres (away from the already calcified front) which eventually "fuse" with each other : new centres ("calcospherites") are formed continuously. This means that as yet unmineralised dentinal matrix at the enamel-dentine junction is separated from the odontoblasts by a layer of mineralised dentine. The first signs of deposition of enamel are the appearance in an extracellular position of "extracellular granules" (FEARNHEAD, 1960: "stippled material", WATSON, 1960). Enamel crystallite growth may commence before the adjacent layer of dentine has mineralised. RÖNNHOLM (1962 B) has expressed this finding in another

Figure 2.29.

Electron micrograph (X 15500) of longitudinal section normal to the surface of the dentine of a pouch young rat-tailed opossum tooth germ, showing the first increment of "enamel" deposited on the surface of the dentine (left hand side).

The surface of the developing enamel is still relatively smooth, and the changes in orientation between neighbouring crystallites within the enamel are very slight. There are no prism boundary planes of sudden change in crystallite orientation and hence no "prisms" in this enamel-dentine junction prism-free domain.

The crystallites are oriented perpendicular to the mineralising front of the developing enamel.

Note the presence of mitochondria in the (inner ends of) the ameloblasts: even within the part of the ameloblasts towards the dentine from the inner terminal bar apparatus, i.e. in that part of the ameloblast which will become known as the TOMES' process when it has completely surrounded itself by "enamel".

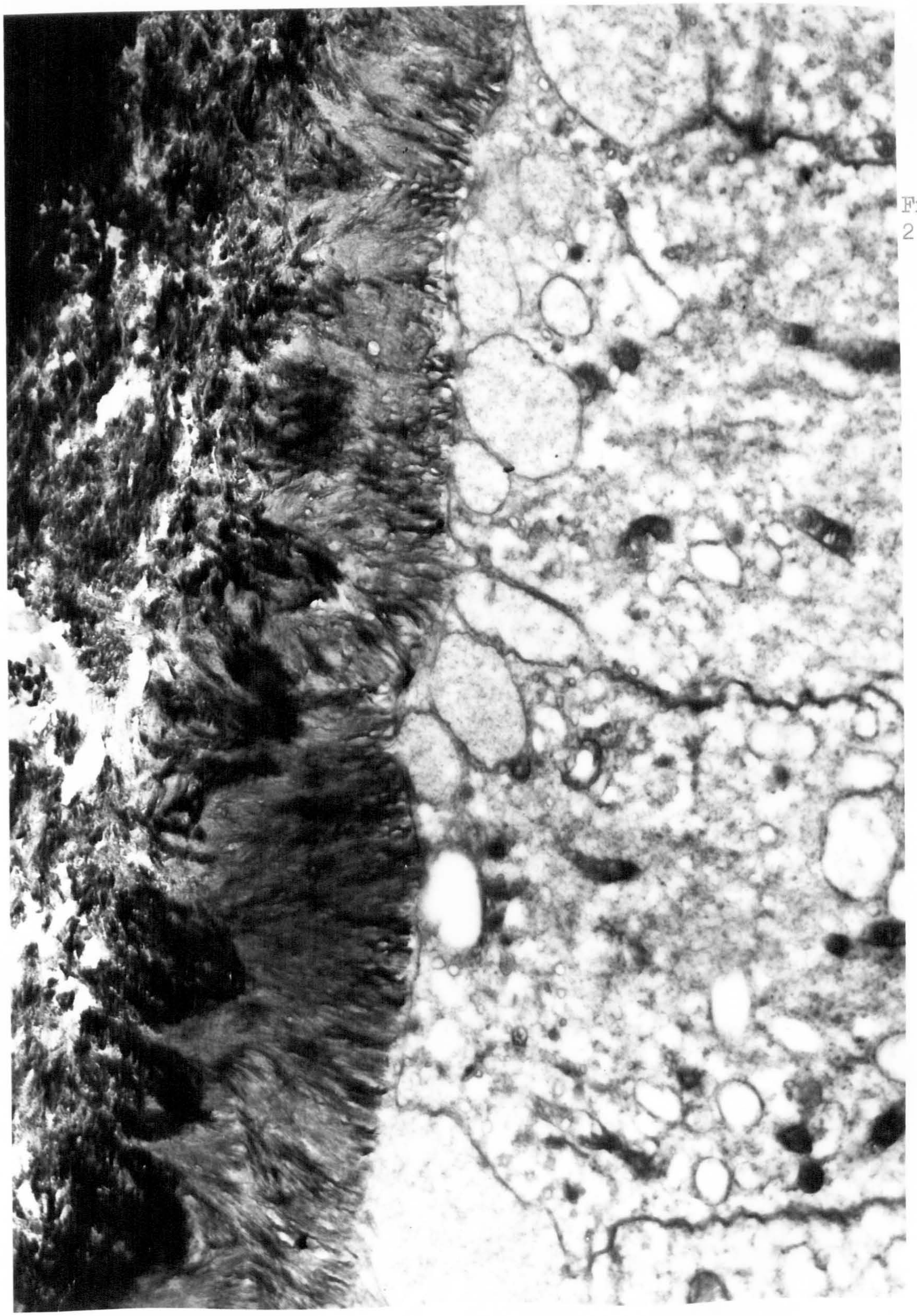


Fig. 2.29.

Figure 2.29. Didelphis nudicaudata: enamel-dentine junction domain. (X 15500).

2.29 way:- "filaments with the cross-banding of collagen are at this stage found together with the enamel crystallites". The mineralized tissue - dentine, and later, enamel - adjacent to the ameloblast becomes smoother during the first formation of enamel crystallites - these appear to fill in the irregularities on the surface of the dentine.

The future enamel-dentine junction may be recognised at the earliest stage, therefore, as the wavy or irregular edge presented by the collagen fibres of the dentine matrix. At a second stage it is the boundary between the extracellular granular material secreted by the ameloblasts and the same collagenous fibres; and it becomes more difficult to trace at third stage, when there is some degree of intermingling between these collagen fibres and the very fine diameter, first-formed, enamel crystallites. The junction is more clearly delineated when the dentinal matrix has mineralised - owing to the great electron opacity of the latter.

The enamel crystallites appear to develop with their long axes perpendicular to the inner-end surfaces of the ameloblasts and the enamel-dentine junction. The very first formed crystallites have an apparently irregular arrangement which reflects the irregular surface of the collagenous matrix of the dentine. This rapidly gives way to a zone of a very few microns in thickness in which the crystallites lie, predominantly parallel to one another, and perpendicular to the now rather smoother surface of the developing enamel (Fig. 2.29). This situation is changed by the appearance of the characteristic topography of the developing front which has already been described. In the one reptile (Caiman sclerops) which was studied the relatively smooth, developing enamel front seemed to persist throughout the formation of the enamel layer; with the crystallite orientation approximating to the perpendicular with this surface (Fig.2.30).

2.4.5. Termination of Amelogenesis - Formation of Surface Zone (Figure 2.31). It is very difficult to prove that the true surface of the enamel has been encountered in a section of a not fully mineralised, developing tooth. The factors which I regard now as indicating that the surface zone has been reached are also to be regarded as results - hence the difficulty.

Figure 2.30.

Electron micrographs of longitudinal sections of Caiman sclerops tooth germ.

Fig. 2.30.1. Low power electron micrograph (X 2780) of longitudinal section of Caiman ameloblasts and stratum intermedium (top). The enamel (bottom) has fragmented, but the original orientation of the crystallites (perpendicular to the surface of the enamel) can be seen in the narrow, intact layer of enamel in contact with the ameloblasts.

Fig. 2.30.2. Higher power electron micrograph (X 21200) of enamel surface, showing the orientation of the crystallites perpendicular to the surface. This orientation is maintained from the enamel-dentine junction (just outside this field) to the true surface of the enamel: there are no "prisms" in Caiman enamel. The ameloblasts have become detached from the surface of the enamel at this particular site.

Figure 2.31.

Electron micrograph (X 17400) of section normal to the surface of maturing Trichosurus vulpecula enamel. In spite of the poor preservation the ameloblasts show some of the changes regarded (by REITH, 1960) as characteristic of these cells during maturation, viz: the presence of large numbers of microvilli and intercellular spaces.

The thin layer of amorphous (granular, grey in the micrograph) material on the surface of the enamel may be organic material re-mobilised from the enamel during its maturation, and/or may represent a precursor stage of the enamel cuticle.

The enamel is rather fragmented (owing to its high degree of mineralisation; this section was cut with a glass knife), but is sufficiently intact to demonstrate the preferred orientation of the crystallites perpendicular to its surface.

Figure 2.30.

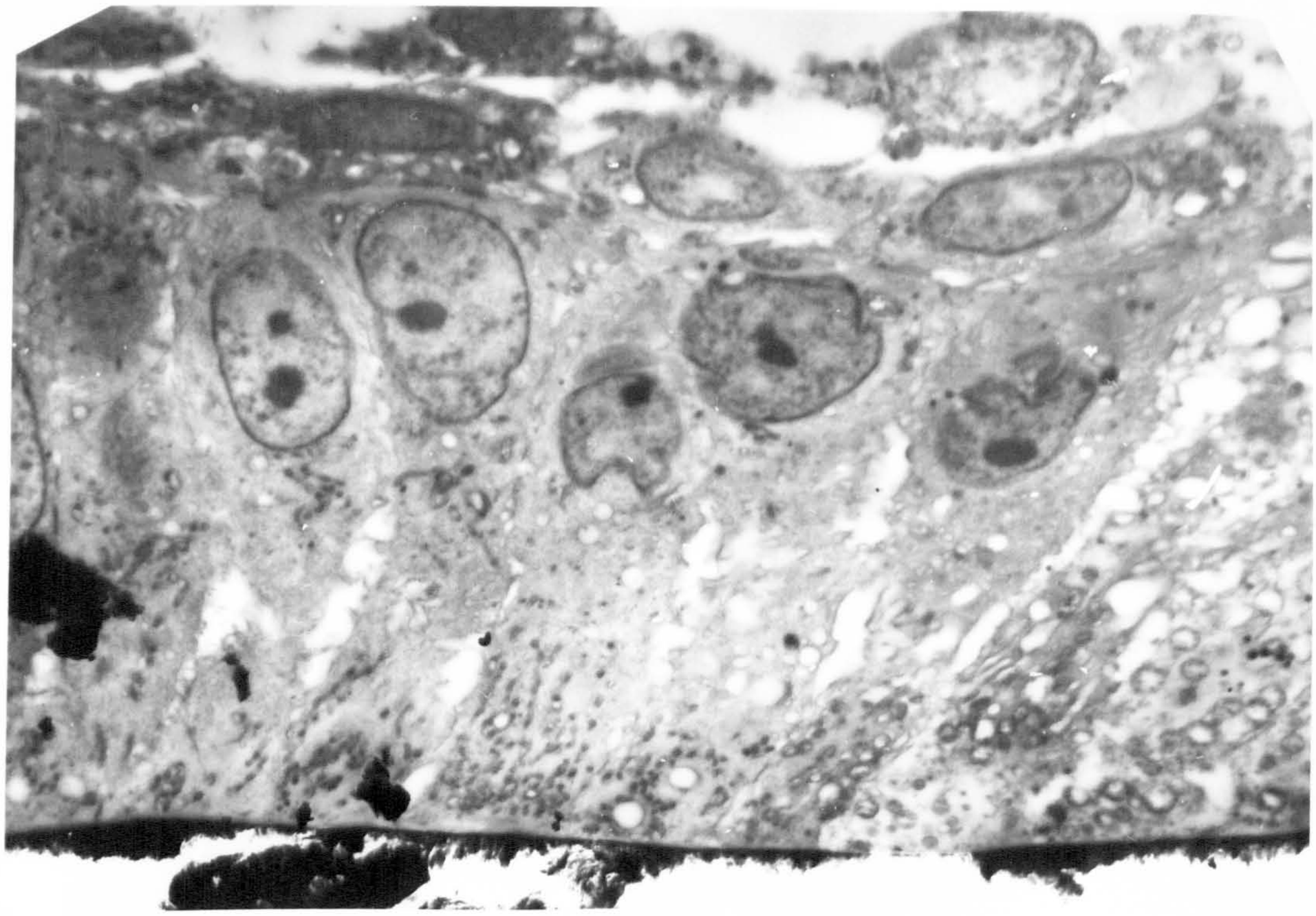


Fig. 2.30.

Fig. 2.30.1. Caiman sclerops: L.S. tooth, L.S. ameloblasts. (X 2780)

Fig. 2.30.2. Caiman sclerops: enamel surface. (X 21200)

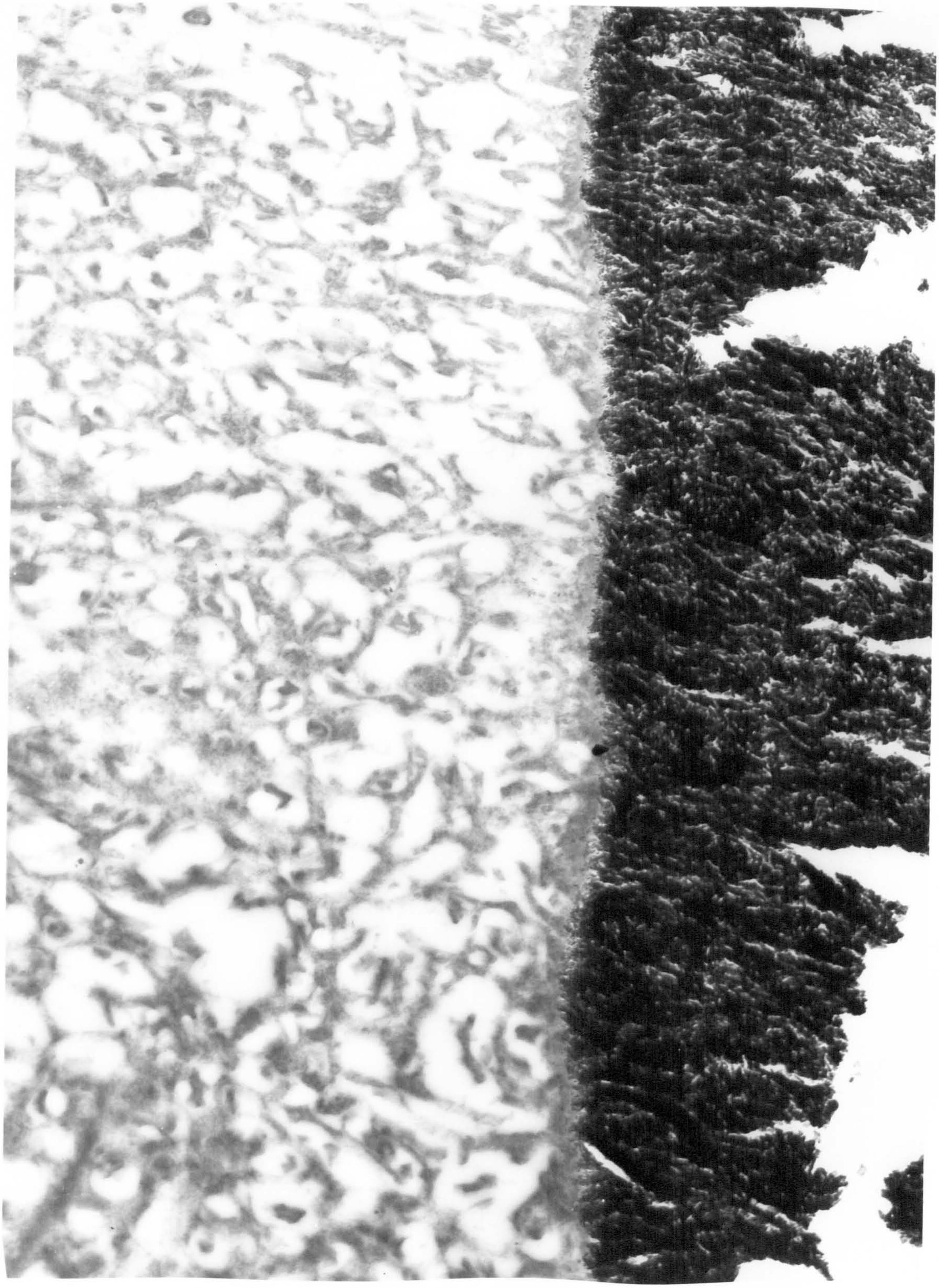


Figure 2.31. Trichosurus vulpecula: surface of maturing enamel and ameloblasts. (X 17400).

2.30 The profile of the true surface of the enamel was found to be smooth; irregularities of a height of more than one micron being unusual. The crystallites c-axes were found to approximate to the perpendicular to the surface and abrupt changes in crystallites orientation were uncommon (Figure 2.31).The true surface zone enamel thus constitutes a single, continuous domain of parallel oriented crystallites. The enamel was much harder closer to the ameloblasts and the crystallites of a greater diameter than those encountered in the ultra-thin sections of the developing, interior bulk of the enamel. So much was this so, that only a very few sections of these regions could be obtained before the glass-knife edges succumbed. The findings of enamel only just soft enough to be cut with a glass knife; larger diameter crystallites and a smoother surface contour were taken together to indicate that the surface zone had been encountered. This indication was confirmed by the presence of intact, shortened ameloblasts in which the nuclear chromatin was "clumped". These "maturing" ameloblasts contained no ∝-cytoplasmic membranes and were separated from each other by largish intercellular spaces, the latter being penetrated by numerous microvilli. (Figures 2.31 and Appendix figures 2.31.2., 2.31.3 and 2.36). These observations confirm those of REITH (1960).

2.5. DISCUSSION

2.5.1. Technical aspects of my own findings

The reasons for regarding the elongated electron dense particles in the "mineralising front" (FEARNHEAD, 1960) as apatite crystallites have been treated thoroughly by RÖNNHOLM (1962). There are, however, good reasons for believing that not all the crystallites show

2.31. up (or show up with equal contrast) in electron micrographs when they are "sectioned" nearly longitudinally. All the crystallites are revealed when sectioned transversely, although not with equal contrast. The contrast of the image of the crystallites is determined by a particular orientation of the lattice planes such that the electron "beam" is diffracted outside the limiting objective aperture and not by the simple mass of crystalline material in the path of the beam (FEARNHEAD and ELLIOTT, 1962). The study of many different planes of section indicates that the crystallites are all exceedingly long. It was therefore considered justifiable to make approximate estimates of the orientation of the crystallites with respect to the plane of section by assessing their degree of "foreshortening". This procedure suffers from the criticism - implied in the previous paragraph - that only crystals with a certain orientation with respect to the plane of section may be revealed; and that the total length of a crystal lying obliquely through the section may not show up with equal contrast. However, the errors inherent in assessing the crystallite orientation from the length of the fragments are not likely to be increased significantly because of this, since large numbers of observations give the impression that the changes in orientation of the c-axes of adjacent crystallites in narrow fields are limited - because of the predominantly parallel array of the crystallites.

The crystallite orientation patterns which were described in earlier sections of this chapter were deduced from observations made on many planes of section and were confirmed in many instances by the study of stereo-pair micrographs in which the pattern could be seen directly. It is therefore only of very limited value to display single electron micrographs and I have preferred to convey my results in diagrammatic form.

The terms "developing"surface of enamel" and "mineralising front" are not synonymous. The difference between the two is the thickness of the layer of amorphous, presumably not inorganic material (extracellular granular material in electron micrographs)between the cell membrane of TOMES' process and the mineralising front. The mineralising front is the plane at which crystals may first be found in electron micrographs. The developing surface is that which is seen

2.32 directly in the scanning electron micrographs (Figure 2.14). The surface of the wax reconstructions (Figure 2.15) represents the surface of the chromophilic (intensely basophilic) material in the developing enamel, and it is not certain whether this does, or does not, include the non crystal-containing portion of the secretion of the ameloblasts. The electron micrographs show that the latter layer is usually very thin (ca. 1,000 Å). The difference in shape between the "surface of developing enamel" and the "mineralising front" is not significant for the purposes of this study.

The shape of the developing surface is of no significance unless both the prisms and the ameloblasts can be related to it. These relationships were determined from the transmission election micrographs. However, the shape of the developing surface could not be deduced from the study of ultra-thin sections alone.

The scanning electron microscope allowed the direct examination of the (conducting film applied to the) developing surface at a resolution of better than 1,000 Å. This could not be achieved by light microscopy because: 1) the complexity of the surface would prevent the successful application of (interference) light microscopical techniques even if the surface were covered with an evaporated-metal reflecting layer: 2) the transparency of the surface would determine that the image "information" came from a thick layer in the surface: and 3) because of the limited resolution of the conventional light microscope. The surface is too soft in the fresh, wet state; and too porous in the (vacuum) dried state to allow of the stripping of replicas to be studied by transmission electron microscopy - this was attempted!

The wax reconstructions demonstrated that all the "holes" which are apparently isolated from the surface in single $\frac{1}{2}\mu$ sections (i.e. in single wax sheets) are connected together; i.e. that the "holes" constitute simple depressions in the developing surface and that none of them are isolated from this surface. The wax reconstructions show crude variations in the shape of the "holes", but the sections from which they were prepared do not reveal the relationship of the individual ameloblasts to the "holes". This was studied in the

electron micrographs, which were in some instances prepared from the ultra-thin sections immediately adjacent to the $\frac{1}{2}\mu$ sections used to make the wax reconstructions. The electron micrographs showed the relationship of the prisms to the shape of the surface of the developing enamel.

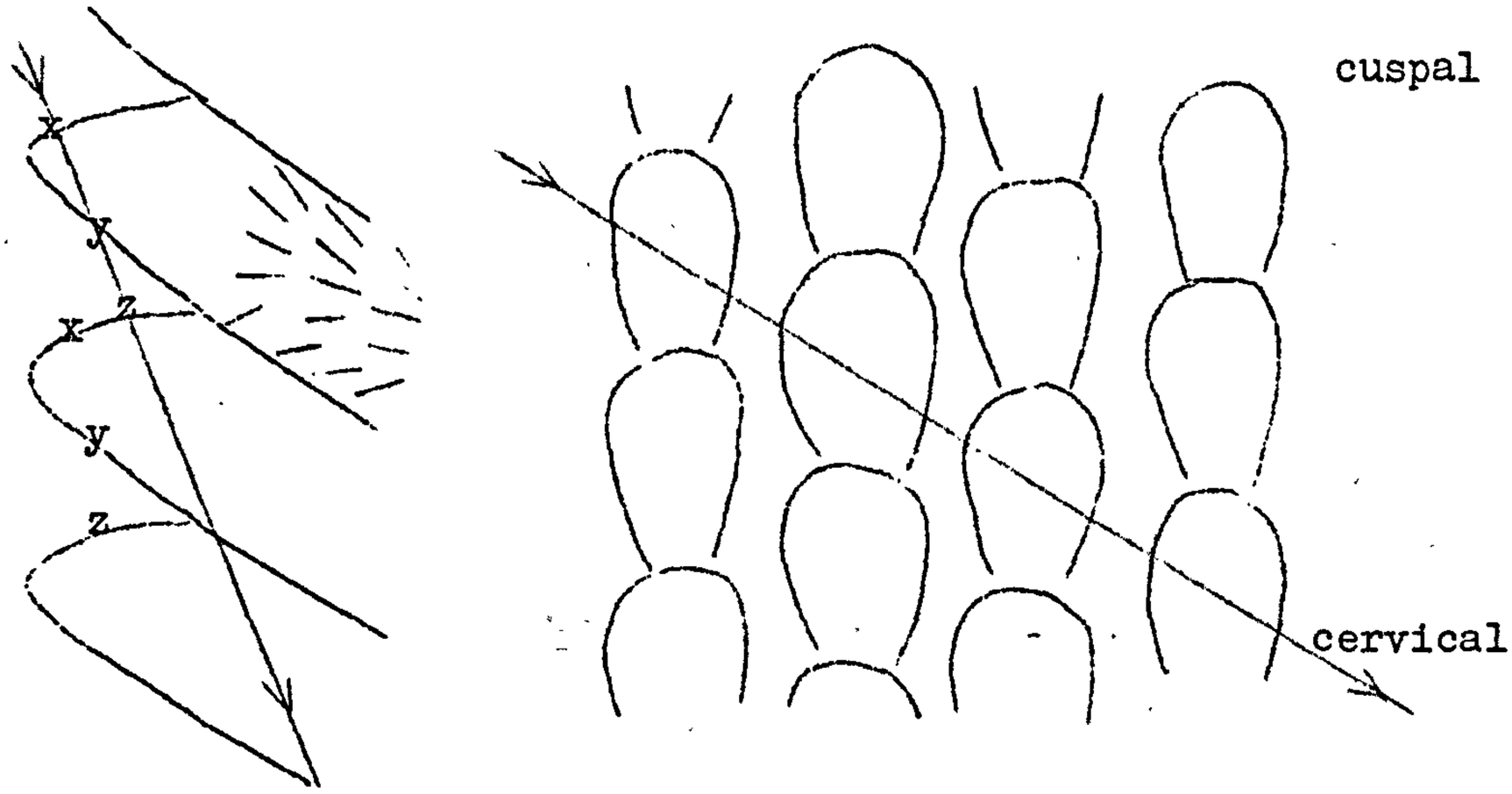
It is abundantly clear that there is no real division of enamel into prism and interprismatic "substances". The regions which can be described by these names are more often continuous with each other. It is not adequate to generalise that these regions differ in the orientation of the crystallites they contain, since we can trace a gradual change in crystallite orientation from one to the other if we start inside the prism near the prism-sheath and work out cervically across the prism, out through the "open" side of the prism, around the end of the cross section of the prism-sheath and via the "interprismatic" region round to the top of the prism sheath again. During this imaginary excursion we meet no barriers; the change in orientation from one enamel crystallite to the next is imperceptible. The really basic morphological features of mammalian enamels are, therefore, only the planes of sudden change in crystallite orientation, i.e. the prism-sheaths of conventional terminology.

"BOXES". Many planes of section which cut the surface of the developenamel obliquely will exhibit the appearance of "boxes" (A-G. GUSTAFSON, 1959). Both transverse sections through a developing tooth and sections cutting the ameloblasts longitudinally may show this profile, which in the case of oblique transverse sections is really just a variety of the "Battlements" profile with some of the clefts bridged over to form the "boxes". It should be noted in this respect that no section can be a true transverse section of the developing tooth, a true longitudinal section of the ameloblasts and at the same time contain the longitudinal axes of the prisms.

The outer surfaces of the lids of the "boxes" appearing in oblique transverse sections may correspond to regions just above or below the points of the picket fence (seen in longitudinal sections) according, respectively, to whether the section enters the enamel surface obliquely from cusp to cervix, or vice versa.

Figure 2.32.

Electron micrograph (X 9900) of oblique transverse section through the mineralising front of developing pig permanent molar enamel. The accompanying diagrams illustrate the plane of section.



The electron-transparent regions within the overall electron-dense "enamel" which were occupied by Tomes' process cytoplasm (the ameloblasts have been removed) and are apparently isolated within the "enamel" are the "boxes". The outer surfaces of the lids of the boxes (x,x,x) are among the most prominent points of the surface of the developing enamel and correspond with the upper borders of the spikes in a "picket fence" (longitudinal)plane of section (x,x,x in diagram). The inner surfaces of the lids of the boxes (y,y,y) are the cuspal surfaces of the depressions in the mineralising front and are related to the cervical regions of the prisms or to the prism boundary planes (the prism sheaths of adult enamel). The floors of the boxes (z,z,z) consist of the crystallites filling in the depressions; this process occurring very predominantly from one (the cervical) side of the depressions.

(The plane of section represented in this figure is by no means the only one in which portions of ameloblast (Tomes' process) cytoplasm are apparently isolated within the enamel, i.e. the "boxes" appearance. In fact this appearance is almost the rule in sections which cut the surface of the developing enamel in other than the "Battlements" or "Picket fence" planes of section defined earlier in this chapter. See for example , figures 2.13.6 and 7.)

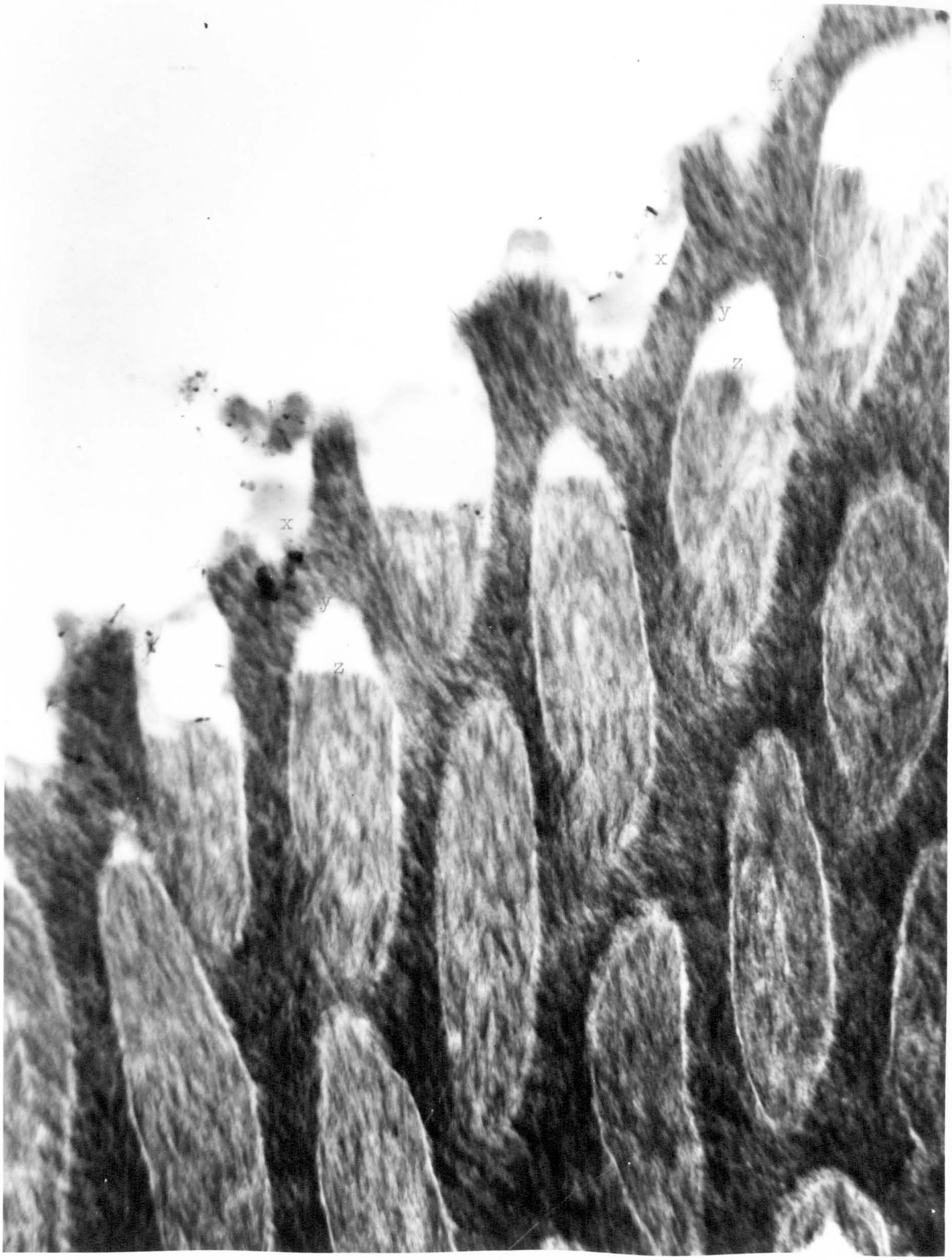


Figure 2.32 Bus: oblique U.S. prisms. (× 9900)

The innermost surfaces of the lids of the boxes are the cuspal surfaces of the depressions in the surface of the developing enamel. (See figure 2.32 and caption).

2.5.2. <u>The cell layers involved in producing enamel</u>. VON BRUNN (1887) showed that the stellate reticulum of the enamel organ of the continuously growing rat incisor disappeared at, or shortly after, the beginning of enamel development. Nobody has shown clearly whether the external enamel epithelium also disappears, or what in fact is the fate of both these cell layers. Every reputable worker has commented on a reduction in the number of identifiable cell layers in the enamel organ, and that the inner enamel epithelium (as ameloblasts; adamantoblasts of LEGROS and MAGITOT (1881)) and the stratum intermedium are always present (thoughout the period of the active production of that portion of new enamel to which they are related). The forming surface of enamel is separated from the odontoblasts by a layer of partly mineralised enamel and a layer of mineralised dentine. One should not give serious credence, therefore, to views of enamel development which implicate the involvement of the odontoblasts and/or other pulpal cells in the elaboration of enamel matrix. (I do not feel that it is yet possible to categorically deny a minor role of the pulpal cells in passing, actively or passively, "inorganic" ions to enamel whilst the growth of its mineral element is yet occurring).

VON BRUNN (1887) also studied the enamel free areas of the crown of the molars of the rat and observed that, although an external enamel epithelium; a somewhat thinner than usual stellate reticulum; and a stratum intermedium all differentiated over the internal enamel epithelium of these areas, the latter cells only reached a length of 26μ as compared with the functional ameloblasts whose length was 40-50μ.

It has not been shown conclusively that macromoleucles may pass between the terminal bars. Until such evidence has been acquired, I would prefer to believe (though I must emphasise that this is a matter of convenience and opinion which helps me to avoid

2.35 the necessity of considering a possible partly-separate origin for the "interprismatic substance") that the presence of the terminal bars means that the potential inter-ameloblastic pathway to the "interprismatic region" in developing enamel is blocked. (MILLER, 1960 has shown that haemoglobin does not pass between the terminal bars of the proximal convoluted tubule cells in mouse kidney). The materials of which enamel is built, whether synthesised in the ameloblasts, stratum intermedium cells or elsewhere, must pass through the ameloblasts and out through the TOMES' processes of the ameloblasts. I shall therefore assume that the principal factors determining the shape of the enamel "prisms" are to be found in the region at and around the secretory poles of the ameloblasts.

2.5.3. Nature of secretory granules in ameloblasts - Unfortunately, the use of only heavy metal fixatives during this study has prevented the study of the natural electron density of the material in the secretion granules of different morphology. Electron scattering depends on electron-electron interactions and this increases with the increasing atomic number (of orbital electrons) of the specimen atoms (hence the use of heavy metal "electron stains"). After osmium fixation the granules have a very variable density. It might prove possible to obtain some information about the nature of the contents of the granules if formalin fixation or freeze-drying were employed: e.g. should any of the secretion granules contain a higher proportion of calcium (Z=20), they would be well differentiated from organic matrix precursor substance granules.

2.5.4. Extracellular nature of enamel formation - the significance of this discovery. There can be little doubt, now, that enamel development is an extracellular process and that the older concepts of the development of its structure must be abandoned. We cannot imagine any longer, for example, that the prisms form by a transformation of the ameloblasts and the interprismatic substance by a transformation of the terminal bar apparatus. All the existing theories of enamel development have proposed separate origins for the prism and interprismatic regions and some have even proposed a third, separate origin for the prism-sheaths, e.g. from the ameloblast cell membranes. All the existing theories must therefore be politely ignored, but

2.36 I would not wish to pass this point without giving all due credit to the sincerely held beliefs and accurately reported observations of the earlier workers. It was their misfortune that the light microscope was and is still not capable of resolving cell membranes. Where the edges or ends of cells can be realised or visualised with the light microscope, it is because of a sudden change in refractive index or in tinctorial affinities between the cell-plasm and its surroundings. The TOMES' process of the ameloblasts is filled with the same secretory product with which it surrounds itself. There is no chemical or physical change (that is detectable with the light-microscope) between the cytoplasm and its surroundings; there is therefore no possibility of deciding, with the light microscope, whether or not the enamel is inside or outside the ameloblast.

I have been priviledged to commence to work in this field at this time; for the important discovery that enamel development is extracellular was made on FEARNHEAD'S part just before I began to work under him in 1959. The implication of this discovery was that all the so-called "different" structures in enamel - the prisms; prism-sheaths; interprismatic substance - develop in the same environment. The mystery was, of course, to discover how one cell can produce three "substances". The solution has lain in re-phrasing the problem.

The problem of explaining the origin of separate inter-prismatic and prism-substances is largely overcome by accepting the evidence contained in electron micrographs of ultra-thin sections of <u>developing</u> enamel, that there is no essential difference between these two "substances". They both contain exactly the same elements: and from the narrow point of view of the electron microscopist those elements are only the crystallites, for nothing else can be "seen"; the organic matrix is "invisible". The problem is then simplified to the extent of only having to explain the origin of the prism sheaths as separate entities. It is only too easy to deny the existence of prism-sheaths after a study of <u>only</u> electron micrographs of undemineralised developing or adult enamel. The "prism-sheath" substance like the rest of the organic matrix in enamel is "invisible" in electron micrographs because of the large difference in the electron scattering power of the apatite crystals and the organic matrix. However, there

2.37 can be no doubt that in decalcified preparations more organic material is retained in these regions than in their surroundings. All that can be said of their development from an electron-microscopic survey is that they develop at the junctions between adjacent domains of crystallites where there is probably a greater possibility for imperfection in the packing of adjacent crystals. Perhaps it is for this reason alone that the prism-sheaths contain more organic material. However, all crystallites end at "prism sheaths": the organic material that is "squeezed"(or in some other way moved) out from between adjacent crystallites can only track outwards towards their narrower (formed later) ends , that is, it must track to the edge of the domain in which it is contained and it must therefore end up at a "prism-sheath" site. This might help to explain the high organic content of the prism sheaths. Now there remains the problem of the changes in crystallite orientation that determine the existence of domains in which the crystallite orientation changes gradually and boundaries at which adjacent domains meet - Why do these changes exist? and how do they arise? It is no less obvious to us to-day that there is a great similarity in the repeat distance of the domains in the enamel and the cells that secrete it, than it was to PURKYNĚ and RASCHKOW (1835) and LINDERER and LINDERER (1837). The repetitive orientating factor must have some connection with the ameloblasts themselves. The results show that the repetitive crystallite orientation patterns in developing enamel are related to the shape of the mineralising front or to the borders of the secretory territories of the ameloblasts (depressions in the developing surface).

The orientation of the crystallites may be controlled by factors related to:1) the ameloblast (TOMES' process) cell membrane: 2) protein fibres (by an epitactic process) which develop with a particular orientation to the cell membranes (perhaps as a result of the "streaming" process suggested by FEARNHEAD, 1961 B):or 3) the "mineralising front" itself. The surface of the TOMES' process is not smooth, but rippled and folded as a consequence of its own secretory activity. The average of a series of positions adopted by the nearest part of the cell membrane of the surface of TOMES' process over a short interval of time. as seen by the developing crystallites in the enamel

2.38 front would probably be smooth. Nevertheless, it does not seem likely that the orientation of the cell membrane directly affects the orientation of the crystallites. Neither does it seem probable or indeed possible that an actively undulating surface of the ameloblasts could determine the orientation of protein fibrils at right angles to itself. FEARNHEAD (1963) has been to some trouble to try to demonstrate fibrils in the substance intervening between the ameloblast cell membrane and the mineralising front of the enamel, but has met with no success.

The third possibility that I raised (there may be many more?) was that the crystallites develop with their c-axes ordered with respect to the plane at which mineralisation commences in the developing enamel front. This front is relatively smooth; has smooth curves; contains the detailed elements of the shape that I wish to hold responsible for the control of crystallite orientation in enamel. In particular, this front contains the very sharp changes in direction which are necessary for the development of the sharp changes in direction of the crystallites in adjacent domains.

The observations which relate crystallite orientation to the mineralising front may be summarised as follows:

1) The majority of crystallites tend to be oriented perpendicular to the nearest regions of the mineralising front; this majority includes all those crystals related to the flattened, cervical sides of the depressions in the mineralising front; and all crystallites in the thin layer of enamel at the enamel-dentine junction, in the surface zone enamel, and in reptilian enamel.

2) The crystallites which are related to the concave lateral and cuspal sides of the depressions in the mineralising front may develop almost parallel with those surfaces; with their long axes almost parallel with the ameloblast long axes.

I consider that all the crystallite orientations that I have observed would be explained by three orientation controlling factors:

1) The crystallites tend to orient themselves (be oriented) perpendicular to the mineralising front. This may be a consequence of their peculiar, very long habit. Their most noteworthy feature is their propensity for growth in length, when and where they are not

2.39 restricted by meeting an obstacle - the obstacle might be either another crystallite at the edge of a field (ie. at a future prism-sheath region) or a deficiency of materials for the continuation of their own growth. J.C. ELLIOTT (personal communication) has suggested that the preferential growth of the enamel crystals along the c-axis - which contrasts with the slight preference for a-axis growth in natural apatites - might be occasioned by their small, but significant, carbonate content. This might either block growth on {100} or encourage deposition on {001} or {111} Anyway, the preferred growth in length of the enamel crystallites would explain the parallel orientation of these crystallites, (at right angles to the mineralising front) if we imagine that some mutual jostling for position is permitted at an early stage.

2) Groups of crystallites, once formed, tend to keep on growing in the same direction.

The fact that groups (RONNHOLM, 1962) of parallel crystallites continue to grow parallel to one another is probably related to their forming into groups (I have expressed this tendency as a preferred orientation of the crystallite a-axes over limited fields). Unless the crystallite orientation is, in fact, determined by the form of the organic matrix, it is possible that the parallel orientation of the flattened hexagon (c-axesparallel; widest sides adjacent) is determined by the balance of mutual repulsive force. The observed distribution of the crystallites would satisfy the conditions for minimum energy in a system in which it is assumed that there is a uniform distribution of charge (of either sign) on the surface of the crystallites; and that the mutually repulsive effect of this charge on the crystallites is not negated by the presence of the organic matrix. However, although it is not possible to decide the nature of the forces which determine the formation of groups of crystallites, it must be assumed that they would render groups of crystallites less flexible structures than individual crystallites. The growth of groups of groups would result in the more parallel orientation of c-axes throughout much longer distances within given domains.

3) An additional "stroking" orienting factor occurs at the surfaces of the mineralising front where there is a relative sliding movement between the front and the ameloblast (TOMES' process) cell

FIGURE 2.33.1

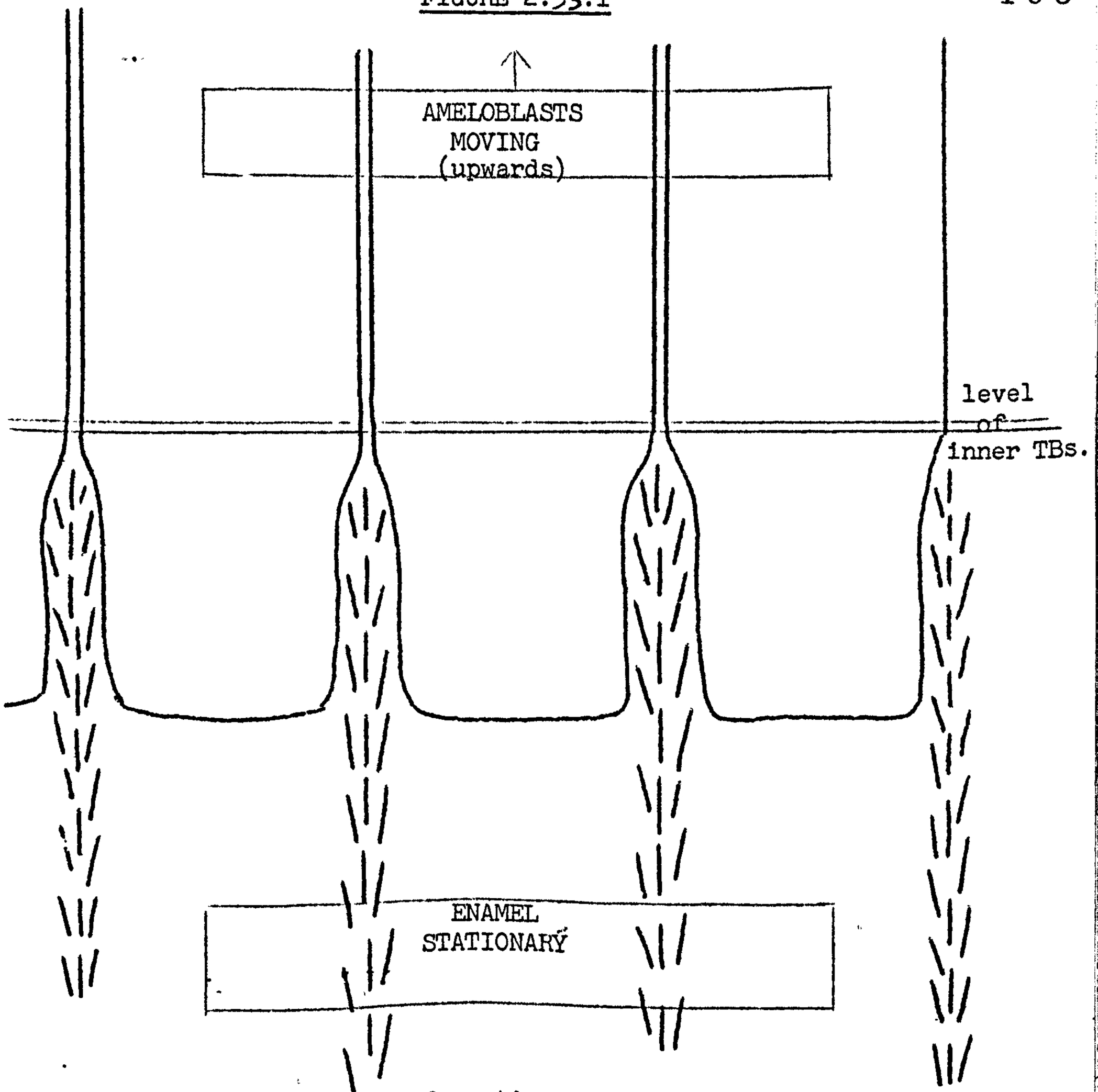
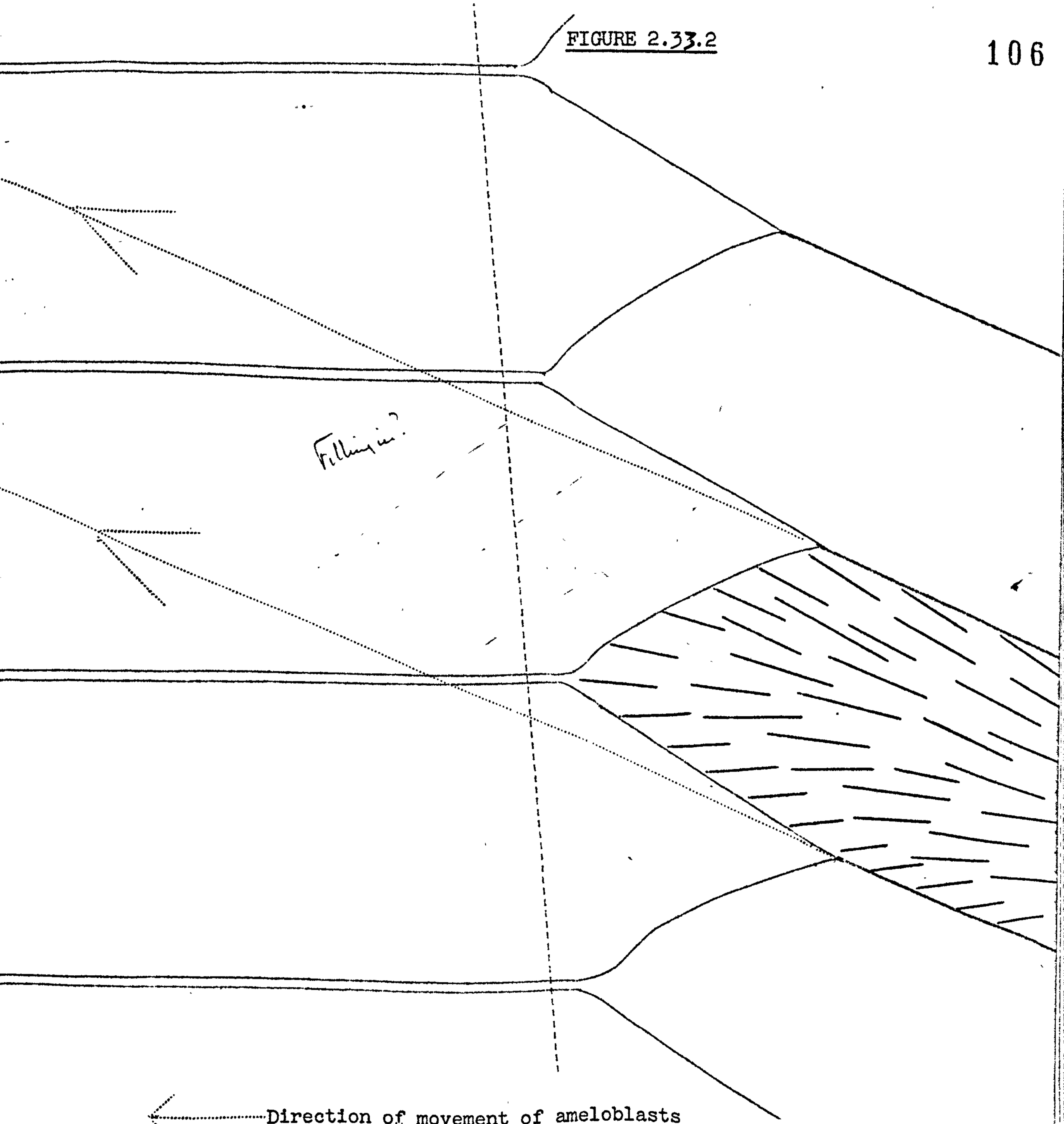


Diagram of Battlements plane of section showing that a relative movement occurs between the lateral surfaces of TOMES' processes and the (lateral) circum-depression regions of the mineralising front: this tends to orient the lateral circum-depression crystallites (i.e. in true inter-prismatic regions in PATTERN 1; in the inter-row sheets in PATTERN 2; or in the "winged process" regions in PATTERN 3) in the direction of this translatory movement. The intra-depression crystallites (which are not shown for clarity's sake) are oriented perpendicular to the mineralising front - there is no relative movement between the surface of TOMES' process and the bases of the depressions in the mineralising front.

This plane of section is highly diagrammatic, since ameloblasts and their secretory territories do not normally lie in the same (transverse with respect to the tooth) axis: except perhaps in Pattern 1 enamel development.

FIGURE 2.33.2


Direction of movement of ameloblasts
Ameloblast cell membranes
Level of inner terminal bar apparatus
Prism boundary plane (prism sheath)

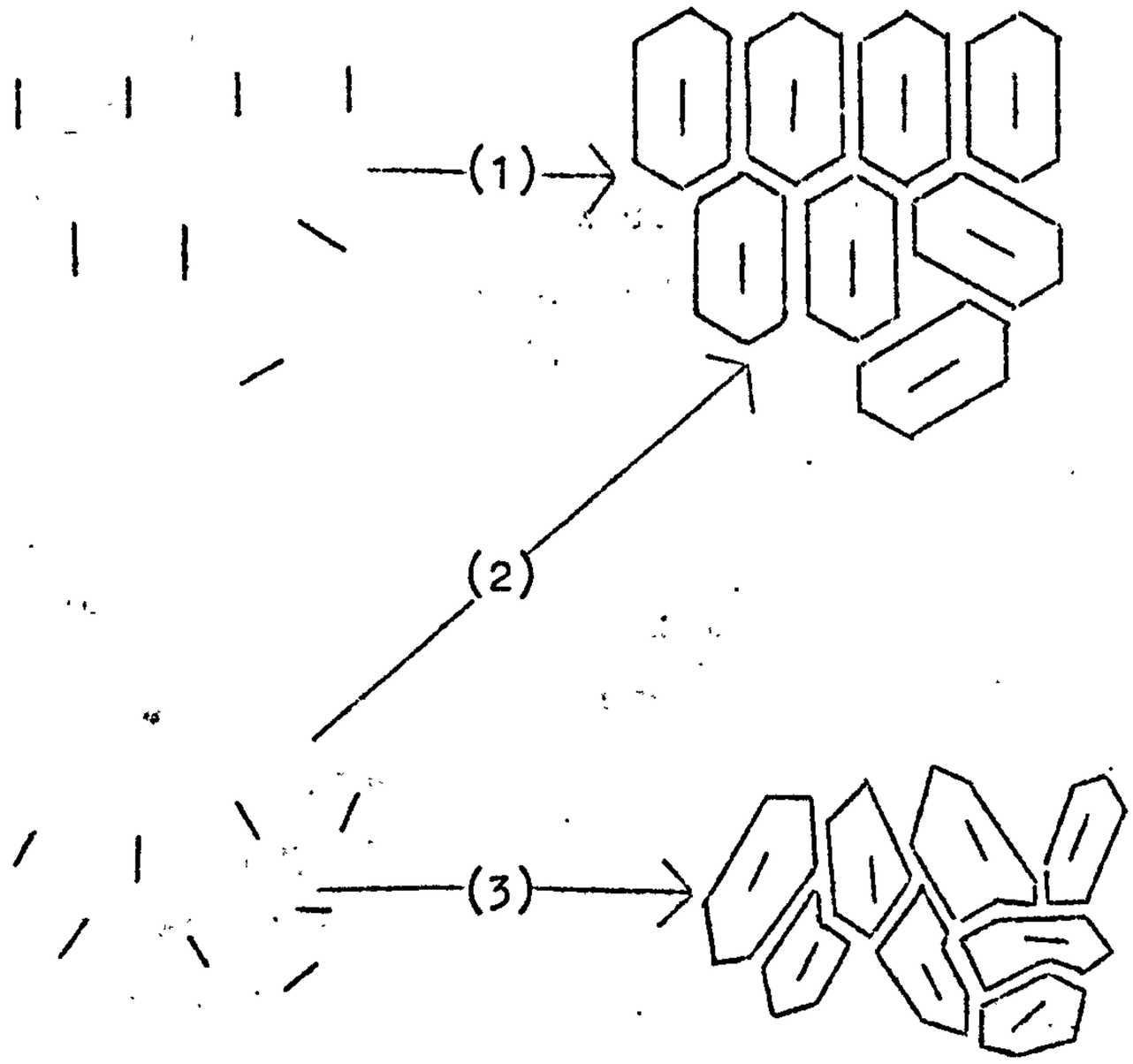
2.40 surface. The crystallites tend to be oriented parallel to the direction of the resultant of this relative movement. Recognition of this factor implies accepting that the environments of the various surfaces of TOMES' process are not entirely equivalent.

The "stroking" orienting factor would explain why the (circum-depression) crystallites in the interprismatic regions (PATTERN 1), interrow sheets (PATTERN 2), or winged process extensions of the prisms in PATTERN 3, deviate from the rule of being oriented perpendicular to the mineralising front, since it is only in these regions that there is a relative, sliding movement between the surface of the ameloblast and the mineralising front (Diagram fig.2.33.)

The existence of differences in crystallite orientation in adult enamel is determined by the existence of different slopes and slants in the plane of mineralisation (i.e. the mineralising front) in developing enamel. If the mineralising front were a plane surface then we should not expect to find any appreciable differences in crystallite orientation. There are two stages during enamel development when this is the case :—

Formation of Surface Zone and first formed layer of enamel at enamel-dentine junction:- The parallel arrangement of the enamel crystallites perpendicular to the surface in the true surface zone; enamel is further confirmation of the generalisation that the crystallites are oriented perpendicular to the mineralising front. The surface-zone enamel develops after the loss of TOMES' processes; i.e., after the loss of the typical topography of the developing front during the formation of the interior enamel. This situation parallels that found in the development of the first increments of enamel at the enamel-dentine junction, before the TOMES' processes and the depressions in the developing front have developed. On the one hand the TOMES' processes have disappeared, presumably as a result of the slowing down of the secretory activity of the ameloblasts; and on the other hand (at the beginning of amelogenesis) they only appear after the secretory activity is really under way. The same situation seems to prevail throughout the development of crocodilian enamel. The relatively flat surface presented by the ends of ameloblasts without TOMES' process

Figure 2.34



Arrow (1) The observed regular grouped (a-axes parallel over limited fields) distribution of the apatite crystals in enamel could be due to a commencement of crystallite growth at fairly regularly spaced intervals.

Arrow (2) Irregularly spaced crystal nuclei could give rise to regularly spaced crystals in the adult enamel if a movement of adjacent crystals due to some mutual interaction can occur.

Arrow (3) Otherwise it would be expected that the crystals would have irregular shapes because growth could only proceed on their free (unobstructed by the presence of other crystals) faces.

2.41 projections leads to a relatively flat "mineralising front" which in turn leads to a relative absence of changes in crystallite orientation; and hence the absence of prism-sheaths in the adult enamel. The foregoing explains the development of the prism-free (one domain) enamels in some reptiles (see POOLE, 1956) and the prism-less enamel layer (domain) at the enamel-dentine junction and at the enamel surface in most mammals. All these instances lend support to the view that TOMES' processes form in association with a particular level of secretory activity of the ameloblasts. There is some other evidence (comparative anatomical) that the length of TOMES' process is related to the rate of formation of the enamel; it being much longer and tapering more slowly in those enamels, e.g. rodent incisor enamels, which form more rapidly.

Determination of crystallite size and repeat distance (Figure 2.34). Since all the crystallites in enamel are of remarkably uniform size and are spaced at fairly regular intervals there are a number of interesting alternative deductions which can be made (and between which we must choose) if it is accepted that the size of the crystallites is the result of the achievement of a certain degree of close packing of the crystallites against each other and the organic matrix. Either:
1) the commencement of crystallite formation must occur at very regular intervals in space (Fig. 2.34.1.): or 2) the crystallites must be able to shift (push, jostle) themselves and/or their neighbours during their growth (Fig. 2.34.2.): or 3) the growth of the crystallites must be limited to occurring on those surfaces which are not blocked by being packed against adjacent crystallites (Fig. 2.34.3.). In this case one would expect to find crystallites of irregular form, i.e. deviating from the straight hexagon. It is doubtful that truly kinked or waisted crystallites occur: their rare appearances in electron micrographs are probably related to section cutting and imaging artefacts (respectively). An objection can also be raised to the first alternative viz. that all the published (two-dimensional) electron micrographs of developing enamel show an irregular spacing of the most recently formed crystallites in enamel, and they also reveal no evidence of a further nucleation (or seeding) of crystals deeper in the developing enamel. I therefore consider that the second alternative (viz. that the

2.42 regular spacing of adjacent, parallel crystallites in adult enamel is determined by the permissibility of mutual jostling) is the more likely. However, it is possible that the apparently irregular distribution of the crystallites in the most recently formed enamel (i.e. the mineralising front is a false impression caused by the irregular distribution of crystallites whose orientation is such that they satisfy the necessary Bragg diffraction conditions for them to appear with sufficient contrast in the image.

There are equal numbers of ameloblasts, depressions in the mineralising front of the developing enamel, and prisms in the adult enamel. If "Kionoblasts" (SAUNDERS, NUCKOLLS and FRISBIE, 1942) are degenerating ameloblasts (in which opinion I concur with KEREBEL and GRIMBERT (1958, 1961)) it would be expected that the total number of ameloblasts would be reduced during the formation of the entire thickness of enamel at any one point on a tooth crown: there must therefore be an accompanying reduction in the number of prisms. FOSSE (1964) has reported that there is a reduction in the number of prisms in passing from the enamel-dentine junction to the true surface of the enamel. PICKERILL's (1913) figures for the ratio of the diameter of the prisms at the enamel-dentine junction to their diameter at the enamel surface (1 : 1.83) and the ratio of the areas of these two regions (1 : 1.76) can also be interpreted as indicating that there is a reduction in the number of prisms during amelogenesis. Many earlier workers had believed that there were supplementary prisms, i.e. an increase in the number of prisms from the enamel-dentine junction to the surface (RETZIUS,1837; TOMES, 1848; CZERMAK, 1850; WILLIAMS, 1886; etc.).

The admission that the numbers of ameloblasts and prisms are equal does not in any way imply that one ameloblast is related to only one prism (and vice versa). This may be so, and is so during the formation of PATTERN I prisms (Fig. 1.1), when "interprismatic" crystallites develop around each depression in the mineralising front and prismatic crystallites develop in relation to their bases. However, parts of two prisms (and parts of two inter-row sheets) are related to each depression in the mineralising front in PATTERN 2 prism formation; and parts of three prisms in PATTERN 3 development. N.B. See Figure 2.25.

.43

It is necessary to strike a note of warning against regarding the prism Patterns described in Section 2.4 (Order characteristic features of enamel development) as much more than diagrammatic concepts which help in seeing and describing the differences between these types of enamel and how they develop. For example, the murine incisor inner--enamel pattern was described as a modification of Pattern 2, when the regions between prisms in the same transverse row are comparable with the longitudinal inter-row sheets found in Pattern 2 enamels (N.B. the Ungulates). However, it would be equally suitable to contrast the murine Pattern with Pattern 3, when these domains between prisms in a transverse direction would be compared simply to the winged process regions of Pattern 3 prisms. The description is not as important as understanding the structure and how it develops.

Likewise the distinction between Patterns 2 and 3 is often not very clear. If we consider the case of decussating Pattern 3 prisms - consider the complication introduced by the decussation - it will be seen that what were Pattern 3 prisms filling in from one <u>corner</u> of the hexagonal grid outlining the secretory territories of ameloblasts, must be called Pattern 2 if they fill in from a different side or angle, since this will involve a transition to the hexagon being filled in from one <u>side</u> (rather than from a corner). This creates a very real practical difficulty in describing, or better, categorising, the shape of the prisms at the borders of sharply defined (decussating) zones, as for example, in the enamel of the <u>Carnivora</u>.

2.44. 2.6. SUMMARY (CHAPTER II)

The relationships of the ameloblasts and the prisms to the shape of the surface of developing enamel were determined from electron-micrographs of ultra-thin sections adjacent to the 0.5.μ sections used to make wax reconstructions. The profile of the developing-enamel surface (in 0.5 μ sections of the methacrylate embedded blocks) was traced at light microscope magnifications of 1,500 diameters. Wax sheets were cut following these outlines and were used to build the reconstructions. The surface of developing enamel was also studied directly by scanning electron microscopy (BOYDE and STEWART, 1963).

Examination of many different planes of section confirms that all the crystallites are extremely long. It is therefore justifiable to make approximate determinations of the crystallite orientation with respect to the plane of section by estimating the degree of fore-shortening (length) of the crystallite fragments in electron-micrographs of ultra-thin sections. Direct confirmation of the pattern deduced in this way was obtained by examining stereo-pair electron-micrographs of the same material.

The crystallite orientation pattern can be described in terms of three-dimensional domains in which the (parallel preferred)orientation of the (long) c-axes of the crystallites only changes gradually from one group to the next; and boundary planes at which there is a marked change in orientation. There is a degree of preferred orientation of the crystallites a-axes,in that the "flat" (parallel to the larger diameter) sides of the flattened hexagonal developing enamel crystallites are parallel and adjacent in small "groups"(RÖNNHOLM, 1962). The domains may be bundles of crystallites as in prisms; or sheets , as in interprismatic regions. The shape of the bundles and sheets, and the orientation of the crystallites which they contain, is determined at the mineralising front. The orientation is related to three controlling factors:-

1. The crystallites tend to be oriented perpendicular to the mineralising front. This orientation may well be caused by the habit of the crystallites themselves.

2.45 2. Groups of crystallites, once formed, tend to continue growing in the same (c-axis) direction.

3. An additional "stroking" orienting factor may occur in regions where there is a relative, sliding movement between the ameloblast cell (TOMES' process) surface and the mineralising front; the crystallites are oriented slightly in the direction of this movement.

The planes of discontinuity in the gradual change of crystallite orientation are related to abrupt changes in the orientation of the mineralising front and are equivalent in position to the"prism-sheaths" of adult enamel.

Interprismatic regions can be defined as such in two common arrangements (Figs. 1.1 and 1.2) where there are:-

1. complete, cylindrical (i.e. circular in Fig. 1.1) prism boundaries (planes of sudden change in crystallite orientation). This is the usual situation in the Sirenia, Cheiroptera, Insectivora and Odontoceti (Cetacea) (SHOBUSAWA, 1952) and it also occurs in the cuspal enamel of human teeth.

2. longitudinal rows of prisms (see Fig. 1.2) separated by interrow sheets of interprismatic ""substance"".

The cervical, open side of the "horseshoe" cross-sectional shape of the prism-boundary faces and abuts on to the cuspal, convex, closed side of the adjacent prism boundary. The lateral (closed) sides of the prism boundaries are separated from each other by sheets of parallel-orientated crystallites, the inter-row sheets, which are probably equivalent to the membranous expansions of MUMMERY (1916). This arrangement corresponds with SMREKER's (1905) Anordnung 2. It occurs to some extent in human enamel but the interrow sheets are thickest, and the separation of the rows of prisms best marked in the Ungulata and Macropodidae (Marsupialia).

2.46.

In a third type of arrangement - SMREKER's (1905) Anordnung 1 - the cervical, open side of the "horseshoe"(arcade) cross-section of the prism sheath faces a ""gap"" between two prisms sheaths cervically. This means that there is no abrupt change in crystallite orientation in passing from the centre of the "prism" into the narrow region situated between the two prisms on its cervical side. The ""gap"" is the "winged process" of MUMMERY (1916), the alar proces of CHASE (1927) or the Flügelfortsatz of VON EBNER (1906). The substance of the "winged process" is therefore in direct continuity with the main body of the prism: it is therefore permissible to say that there is no region which can be called interprismatic. This arrangement (Fig. 1.3.) is the one most commonly found in human and elephant enamel (SHOBUSAWA, 1952).

The arrangement depicted in Figure 1.4. is that found in murine (Murinae-Rodentia) incisor inner-enamel. The longitudinal interrow sheets (of Fig. 1.2) are interrupted by the decussation of alternate transverse rows of prisms. The prisms of adjacent zones (layers; lamellae; HUNTER-SCHREGER bands; para- and diazones) cross each other. Ameloblasts must therefore be able to slide past each other (and in opposite directions in alternate rows during the formation of the incisor (inner or plexiform layer) enamel in the Muridae and Sciuridae). Terminal bars have been found at both ends of the ameloblasts. Whatever the forces of attraction between adjacent cells at the level of the terminal bars, they must be such as to allow a sliding movement of the adjacent cell surfaces past each other in a horizontal, but not a vertical direction.

The prisms of alternate zones fill in from alternate though not exactly opposite sides. It is not known whether this is the cause or the effect of the movement of the ameloblasts which must occur during the formation of the zones. This point is discussed in Section 8.2.

SCANNING ELECTRON PROBE X-RAY EMISSION MICROANALYSIS OF DEVELOPING (MATURING) ENAMEL.

3.1. Introduction

Enamel is a soft, highly organic, poorly mineralised tissue upon first being secreted, and it gradually acquires a high mineral content whilst losing a proportion of its organic content (HERTZ, 1866). HOPPE (1862) observed that young newly deposited enamel could be charred, whilst adult enamel remained almost white after heating. The earliest observers knew that it was soft at the first stage of development.

HOPPE (1862) showed that young enamel is positively birefringent: adult enamel negatively birefringent, which results were confirmed by VON EBNER (1890, 1903). VON EBNER (1903, 1906) related the sign of the birefringence to the (crudely-assessed) hardness of the developing tissue. SCHMIDT (1924) confirmed HOPPE'S and VON EBNER'S observations of the change in sign of the birefringence.

The current concept of enamel maturation is based upon a number of studies of "The pattern of progressive mineralisation in Human Enamel".* (ENGFELDT, BERGMAN and HAMMARLUND-ESSLER , 1954; GRABB and DARLING, 1956, 1958, 1960, 1962; HAMMARLUND-ESSLER, 1958; ALLAN, 1958; CRABB, 1959; AVERY and VISSER, 1960; AVERY, VISSER and KNAPP, 1961; and AVERY, 1963). These studies have been collated by CRABB and DARLING (1962)*, who summarised the findings as follows:- (1) the enamel matrix is mineralised (to a small extent) shortly after it is laid down. (2) A higher degree of mineralisation then becomes apparent in the enamel at the enamel-dentine junction. (3) The zone of mineralisation spreads out from the enamel-dentine junction towards the enamel surface, beginning first over the cusp tips and subsequently spreading cervically. (4) The surface zone mineralises considerably in advance of the underlying enamel. (5) The process of mineralisation does not follow the incremental pattern of the matrix as thought by some previous workers.

3.2 3.2. Scanning Electron Probe X-ray Microanalysis. The characteristic x-ray spectrum emitted by a specimen surface under electron bombardment in a scanning electron-probe microanalyser can be used to (1) form an image by all the x-rays emitted, i.e. an image formed by the "white" radiation: (2) to form an image using a selected x-ray wavelength characteristic of a given element, and thus to show the distribution in the surface of a given element: (3) to provide an analysis of the elements in the specimen; either in the whole surface, with the electron-probe scanning; or in a given spot, with the electron-beam stationary; using an x-ray spectrometer, counting and recording equipment: and (4) likewise to chart the distribution of a selected element along any given line (of any width from that of the probe, to the width of the scan) across the specimen surface.

We have used a Cambridge Instrument Company "Microscan" (scanning electron probe x-ray microanalyser) to measure the CaK_{α} emission from developing enamel. The problems which are associated with the use of this method and which are peculiar to the study of dental tissues have been described by BOYDE, SWITSUR and FEARNHEAD (1961), BOYDE, SWITSUR and STEWART (1962), BOYDE and SWITSUR (1962) and SWITSUR and BOYDE (1962). The basis of the method is treated in references appended to these papers.

Specimen Preparation. The specimens used in this investigation were two developing human mandibular third molars (extracted from 8-11 year old children for othodontic reasons) and two 2-day old rat mandibular molars. The enamel-organ and pulp were stripped off: the teeth dehydrated in graded methyl alcohols and embedded in methyl methacrylate. Longitudinal ground sections were prepared after a prolonged polymerisation at 40°C. The ground sections were mounted alongside fragments of Apatite* (Durango, New Mexico), using a cold-cure acrylic resin. The surfaces to be examined were polished on metallurgical polishing papers down to grade 4/0 (John Oakey and Sons, Wellington Mills, London, England) and covered with a layer of aluminium

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* Supplied by R.F.D. PARKINSON, Doulting, Shepton Mallet, Somerset, England.

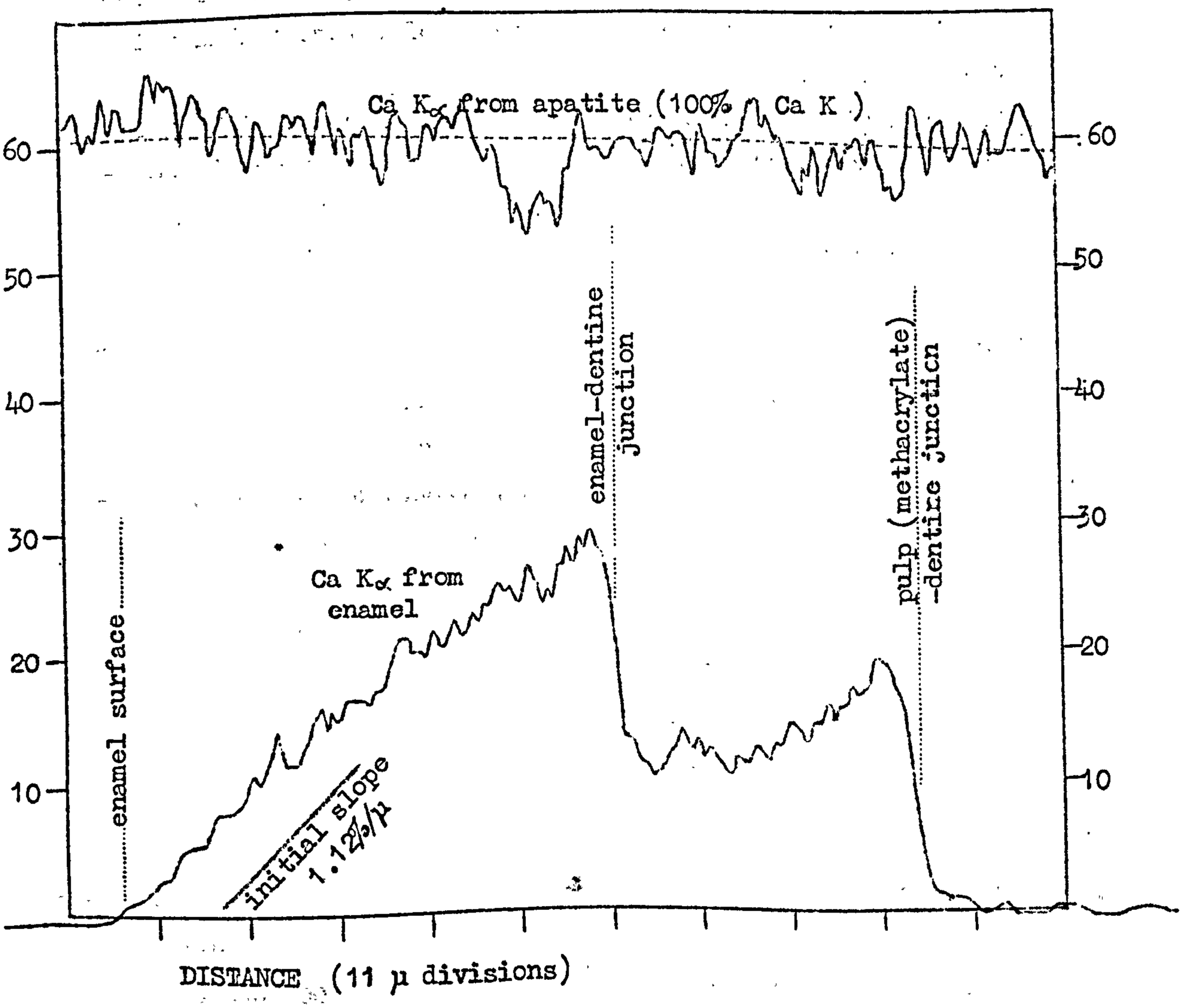
3.3 (approximately 300 Å thick) by evaporation in vacuum. The specimen surfaces were photographed (in reflected light) in order to facilitate the identification of areas of interest whilst in the Microanalyser.

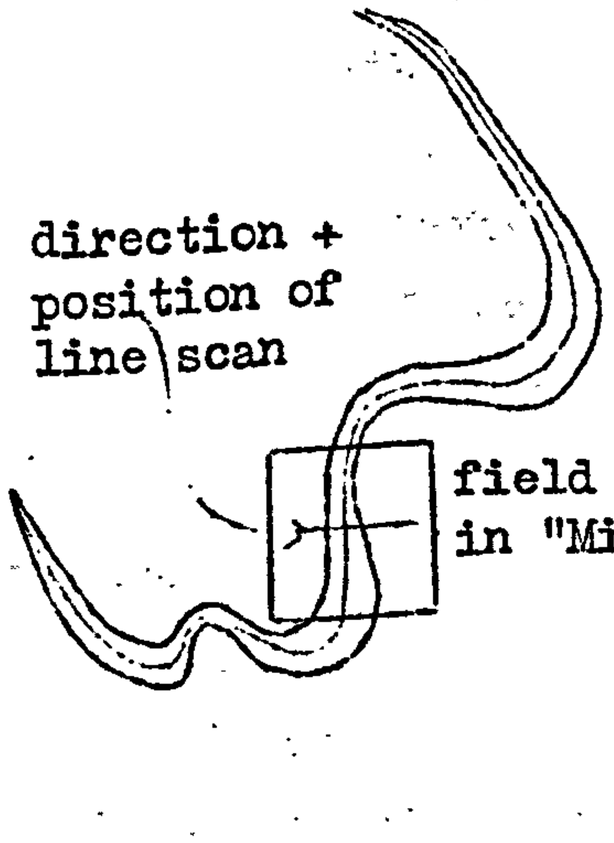
Calcium Analysis. These specimens have been examined with a view to obtaining more accurate quantitative analyses of the mineral content of enamel during its development (maturation); and by a less questionable method than either x-ray absorption microanalysis as practised in contact microradiography or intrinsic birefringence measurements using the polarising microscope. The elements in the mineral component of enamel which can be analysed by the methods under consideration in this section (and available at the moment) have atomic numbers $Z > 12$, i.e. Calcium ($Z=20$) and Phosphorus ($Z=15$). We have confined our observations to those which would enable us to deduce the calcium content.

DEAKIN'S and BURT (1944) reported that the calcium and phosphorus contents of pig enamel increased linearly and in constant ratios to each other during the maturation of pig enamel. Many publications have asserted the close similarity between the mineral content and hydroxyapatite. The proportions by weight of Ca and P in the natural mineral apatite (largely fluor-apatite) are insignificantly different (for our purposes) from the proportions in hydroxy-apatite. We have therefore felt justified in using samples of naturally occuring appatite as standards for our 100% CaK_{α} or 100% apatite level. It should be noted that chemical analyses reveal that enamel mineral is not pure hydroxy-apatite (Ca:P ratio 2.16 by weight). Assuming a uniform Ca:P ratio of 2.05 (after ANGMAR, CARLSTRÖM and GLAS, 1963) for the enamel, a correction factor of x 1.05 could be applied to express the results as "percentage of enamel mineral".

Our interest has been to obtain accurate values of the average mineral content in the enamel at increasing distances from the developing enamel front. We have not been concerned to obtain the analyses from small volumes; so that in order to avoid the errors (due to a poor specimen surface finish) which can occur when a static probe is used (when, for example, the emitted x-rays may be "shadowed" off from the x-ray spectrometer by an irregularity in

Figure 3.1. Ca K_α emission from the surface of a longitudinal section of a rat mandibular molar.





The top trace is of the Ca K_α X-ray emission recorded from the apatite standard: the fluctuations are due mainly to the roughness of its surface (see text).

The bottom trace records the Ca K_α emission from a 20 μ-wide line scanned across the thickness of the occlusal region of the tooth shown in the accompanying diagram.

The side scale divisions representing X-ray counts per unit time are arbitary (1 division = 10 X-ray counter pulses per second. Integrating time constant = 0.44 seconds: notice how the trace is smoothed out by using a longer time constant in Fig. 3.2.).

3.4 the surface) we have used a probe scanned rapidly in the x direction across a line (y direction) some 20μ wide; whilst moving the scanning probe very slowly along a line in the y direction. The analysis thus obtained is an average of the mineral content ($CaK\alpha$ radiation emitted) in a line some 20μ wide. The statistical reliability of the results is increased as the analyses are based on an increasing count of characteristic x-ray quanta. This has been achieved by recording the x-ray emission over longer periods, ie. by using much longer scanning periods than is normal. Some of the "wide-line-trace" analyses have taken up to 2 hours to record from a line only 1 mm. long on the specimen surface. The slow-scan allows a long integrating time constant to be used. (Time constant = time over which x-ray counter pulses are averaged-out to derive the potential which operates the pen-recorder). This means that the fluctuations of the graph-line on the pen recording are of reduced amplitude, i.e. the the mean probable error is reduced.

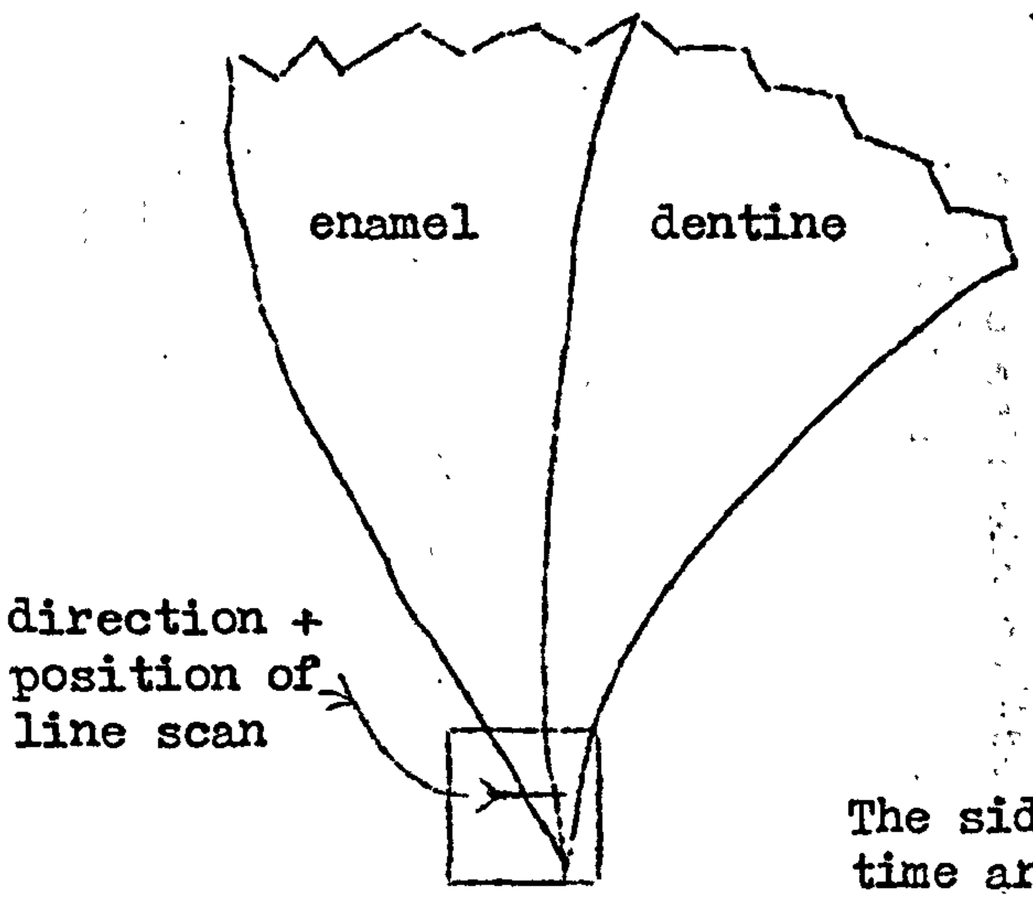
The specimens have been oriented in the Microscan so that the (y-axis of the) scan crossed the developing surface of the enamel at right angles .

One of the defects of the scanning electron-probe method is that the x-ray crystal spectrometer is only "focussed" on one line of the specimen surface. Movement of the electron probe to either side of this line is equivalent to a relative movement of the x-ray source and the spectrometer; and if the crystal was adjusted to reflect the maximum number of x-rays of a particular wavelength into the x-ray counter, the number will fall off on either side of the optimum position. In effect, an artefactual change in concentration of the element of interest(for which the spectrometer is set up) will be recorded, due to "defocussing" of the spectrometer. The slow scan analyses were done in a direction parallel to the line of focus of the crystal of the spectrometer in order to minimise the defocussing effect on the spectrometer. The remaining defocussing effect (and any other effects which can cause a variation in the count rate from a uniform sample as the probe is moved some distance over its surface) were discounted by first taking a trace from the specimen; then placing the standard in the position previously occupied by the specimen and taking another

Figure 3.2. Ca K_α emission from the surface of a longitudinal section of a developing human mandibular third molar.

The top trace is of the Ca K_α emission from the apatite standard, showing the fluctuations in X-ray counts recorded due principally to the poor surface finish.

The bottom trace records the Ca K_α emission from a 20 μ-wide line scanned across the edge of the developing enamel in the cervical region of the tooth (arrow in inset diagram).



The side scale divisions representing X-ray counts per unit time are arbitary (50 = 500 X-ray counter pulses per second. Integrating time constant = 9.6 seconds).
The bottom scale divisions show the distance scanned across the specimen: 1 division = 11 μ.

Ca K_α X-ray emission

Ca K_α from apatite (100% Ca K_α)

(dentine)

Ca K_α from enamel

enamel-dentine junction

initial slope 4.37%/μ

enamel surface

DISTANCE (11 μ divisions)

3.5 trace under exactly similar conditions. The practical result of this procedure is that the 100% CaK_{α} levels on the pen traces (Figs. 3.1., 3.2.) are not parallel to the abscissa - they have been drawn in as separate lines. The 100% CaK_{α} level is that recorded from the apatite standard in each case.

3.3. Results. The analyses (ROSSER, BOYDE and STEWART, in preparation) show that there is a smooth increase in mineral content in the enamel from the developing surface inwards towards the enamel-dentine junction in both the human and rat molar teeth studied. The CaK_{α} emission from the rat molar enamel (Fig.3.1) reached a maximum value close to the enamel dentine junction. In the human enamel the CaK_{α} emission rose steadily from the developing surface; then sloped off to a plateau; and this level was maintained until close to the enamel-dentine junction (Fig. 3.2). The irregularities in the CaK_{α} traces were almost certainly caused by irregularities of the specimen surfaces. The polish achieved on these specimens was not as good as had been achieved on earlier, fully mineralised, adult rat and human enamel specimens (BOYDE, SWITSUR and FEARNHEAD, 1961).

3.4. DISCUSSION

3.4.1. Present Results.

The terms "calcification" or "maturation" or "mineralisation" do not connote quantity or direction. I shall therefore introduce the concept of iso-concentration-contours in discussing the pattern of mineralisation of enamel.

The present (X-ray emission microanalytical) results are certainly not comprehensive enough for any comment to be made on the pattern, and changes in the pattern, of the iso-Calcium-content contours during the mineralisation of all the enamel. Our results have been restricted mainly to the occlusal enamel of two rat molars and the cervical enamel of two human third molars. However, the nearest approximation that could be derived from our data is that the iso-Ca-content contours are parallel to the incremental lines. It is certain that they are nowhere near parallel to the transverse axis of the tooth. (DIAMOND and WEINMANN, 1940; GLOCK, MELLANBY, MELLANBY, MURRAY and THEWLIS, 1942; ORBAN , 1957). It also seems

3.6.

that the ("iso-concentration-jump value") contours are almost equally spaced, i.e. that there is no second, sudden increase in the rate of increase in mineral content as was suggested by DIAMOND and WEINMANN (1940). The amplitude modulations of the CaK_{α} emission pen-recording traces (figures 3.1., 3.2.) may reflect real variations in the mineralisation of successive increments of enamel (such as were noted by AVERY, 1963) but cannot, at the moment, be distinguished from "NOISE". The present results provide no evidence of the layer of higher mineral content at the true surface of the enamel (ie. after the full thickness of the enamel has been deposited) that has been reported by most workers (in man - CRABB and DARLING, 1962; and rat - SUGA and GUSTAFSON, 1963). This is probably simply because the enamel examined in the present study was still being formed. Thus we have not examined "the second phase of mineralisation (which) starts in different places and at different times in each zone" in rat molar enamel (SUGA and GUSTAFSON, 1963).

A number of effects could contribute to the final (concave) shape of the CaK_{α} traces (Figs. 3.1., 3.2.) at the foot of the slope where the electron-probe first crosses on to the surface of the developing enamel:- (1) The 20μ wide line scanned by the probe may not have been oriented perpendicular to the surface of the enamel in spite of the care taken to ensure that this was the case: (2) The edge of the surface of the developing enamel may not have been exactly perpendicular to the surface of the specimen - care was also taken to ensure that this could not be a serious critisism: (3) When the probe first crosses on to the calcium containing part of the specimen, a number of electrons may diffuse out into the adjacent non-calcium continaing region (- still possessing energies greater than the excitation energy for CaK_{α}): (4) CaK_{α} radiation may be excited in the"enamel" by continuous radiation excited by the electron beam in the embedding medium. The magnitude of these effects is however, likely to be exceedingly small. The major part of the slope of the CaK_{α} traces is surprisingly linear in both (human and rat) cases. However, a much greater length of the final part of the slope is convex in the human.- where it levels off to a plateau before the

3.7 enamel-dentine junction. This difference is probably only related to the difference in the degree of mineralisation reached in the most-highly mineralised regions in these specimens. In the human specimens, the Ca-concentration (at 91% approximately) would be nearing the final value (96%) whereas the rat enamel specimens had only achieved approximately a 65% Ca concentration. The slope is still linear at the 65% Ca level in the human case.

(The observation of a lowered mineral content in the dentine just inside the enamel-dentine junction in the developing rat molar parallels the observations by HAMMARLUND-ESSLER (1958) of a similar zone in human dentine).

The histochemical study of HINTZSCHE and BAUMANN (1933 - they found a dense reaction for Ca and P in the enamel nearest to guinea-pig ameloblasts); the E/M identification of apatite crystallites in the enamel very close to the ameloblasts; and the present x-ray microanalytical results make it quite clear that enamel contains a mineral component (almost) from the first moment that it is secreted and that the amount increases gradually at any one site in the enamel thereafter, until a certain maximum concentration has been reached. This concept is in contradistinction to the more current one which uses the phrases "calcification" of the enamel "matrix". The material which has been called "matrix" by previous workers is partly mineralised - more accurately, it already contains (apatite) crystallites.

The concept of a matrix, and/or of a zone of newly formed enamel which was entirely organic, grew up with the use of particular methods, viz:— 1) Birefringence measurements to assess the mineral content - KEIL (1935), HARDERS-STEINHÄUSER (1938), and many others (see CRABB and DARLING, 1962). The most recently formed crystallites are far too small to have any effect on visible-wavelength radiation: it is not possible to defect their presence and it is certainly not possible to measure their intrinsic birefringence. The use of intrinsic birefringence measurements to assess mineral content is open to serious

3.8 criticism (VON EBNER, 1906; ALLAN, 1959; HELMCKE, 1959 A, B; TORELL, 1960 A , B; CARLSTRÖM, 1960; ANGMAR, CARLSTRÖM and GLAS, 1963):

2) Decalcification of developing teeth to retain the organic matrix. There is no correlation between the (crudely-assessed) retention of stainable organic matrix and mineral content (CRABB and DARLING, 1962). The method was used by HERTZ (1866), CHASE,(1935), DIAMOND and WEINMANN (1940) and MARSLAND (1952):

3) Mass per unit area measurements via x-ray absorptiometry - (microradiography) (References in Section 3.1; CRABB and DARLING, 1962; and AVERY, 1963). There are very few reported instances of the study of the mineral content in developing enamel using quantitative contact microradiography (using a calibrated step wedge - HAMMARLUND-ESSLER, 1958; CRABB and DARLING, 1956). The complete photographic contrast range (white-black) does not normally cover the complete mineral concentration range (100%-0%) and the photographic "information recording" method is very insensitive towards the extremes of its range. This has meant that the mineral content in first-formed enamel has gone undetected and that the first stages of its increase have been mapped with great inaccuracy:

4) Autoradiographic studies reveal both the uptake of new inorganic ions on growing crystal surfaces and the exchange of ions on already formed surfaces. Again, a photographic recording (counting) procedure is used and this is too insensitive to contain the enormous contrast between the number of radioactive atoms deposited in the mineralising front and the far greater number in the underlying bulk. Thus LEBLOND, BELANGER and GREULICH (1955) and KUMAMOTO and LEBLOND (1956) found no deposition of P^{32} or Ca^{45} in "pre-enamel".

All the four methods just mentioned suffer from the disadvantage in practice that they cannot both analyse the mineral content in the first formed enamel and that in the more "mature" tissue, without giving rise to the impression that there is a rather sudden increase in the rate of increase in mineral content somewhere between the surface of the developing enamel and the enamel-dentine junction.

A loss of organic material from enamel during maturation has been reported by DEAKINS (1942) and EASTOE (1960, 1963). HAIS (1953)

3.9 studied the maturation of enamel using flouresence microscopy - a reduction in flouresence corresponding to a reduction in organic content of the enamel. He found that maturation progressed from the enamel-dentine junction towards the surface; i.e. very approximately following the incremental lines. KARPISHKA, LEBLOND and CARNEIRO (1959), YOUNG and GREULICH (1963) and EASTOE (1963) have suggested that a re-distribution of enamel organic matrix occurs, perhaps as a result of the increasing deposition of the mineral component. It is difficult to understand how a highly-ordered fibrous protein structure - the existence of which is implied in an epitactic concept of enamel mineralisation - could be broken down to be re-mobilised: EASTOE (1963) has suggested the concept of thixotropic properties in a gel-like organic matrix in order to explain its observed behaviour.

RÖNNHOLM (1962, p. 274 Chart I) reported that the increase in crystallite "thickness" (up to 170 Å) with distance (up to 35μ) from the mineralising front was linear. He reported a maximum average crystallite "thickness" (as against"width") of 300 Å in the surface enamel of "adolescent" teeth. These figures mean that the increase in "thickness" with distance from the mineralising front was linear at least until 25% of the final mineral concentration had been reached (more than 25% if the crystallite "thickness" increases more rapidly than "width"; which it certainly seems to do, since the more mature enamel crystallites are more nearly perfect hexagons - HÖHLING, 1960). A linear increase in diameter would imply a square law increase in mass of mineral if the new growth occurred equally on all the crystallite {100} surfaces. RÖNNHOLM'S observations definitely still apply to a zone in the enamel which is well away from the concave foot of the slope of our CaK_{α} emission traces; ie. they apply to a region in which the present results indicate a linear mass increase. If both RÖNNHOLM'S observations of crystallite thickness and ours of mineral content are to be taken at their face value, it would imply that there is a very marked tendency for the crystallites to grow in "thickness" instead of "width"; ie. for them to grow only on those surfaces which would be associated with a change of cross-sectional shape from a flattened hexagon to an equal-sided hexagon. However, RÖNNHOLM'S (1962)

3.10 measurements of (extremely narrow) crystallite diameters direct from electron micrographs are likely to be in error for reasons which are considered in Chapter 10; NYLEN, EANES and OMNELL (1963) considered that a uniform rate of growth on all surfaces is indicated in rat enamel crystallites;and errors associated with the present method deserve prominent mention. The distance to which the electrons penetrate the specimen surface with sufficient energy to excite the characteristic CaK_{α} radiation depends on the density of the specimen at that point. This means that x-rays will be excited from a greater volume of less dense, less mineralised tissue - and comparable volumes will not be analysed. The net result will be to increase the apparent Ca concentration in the less mineralised regions - this would tend to iron out the concavity at the foot of the slope and would shift the curve away from the direction predicted by RÖNNHOLMS figures. However, this effect is "balanced"to some extent by the greater absorption by the specimen of x-rays excited at a greater depth in the specimen (ARCHARD and MULVEY, 1963).

A linear increase in crystallite diameter - square law increase in mass - with increasing distance from the developing surface would mean that the rate of increase in mass of the crystallites was directly proportional to their surface area - if all (100) faces grow equally. Our results do not support this concept and would suggest that certain growth-rate limiting factors come into action. One of these factors might be the limited availability of ions due to diffusion gradients or to some influence of the surrounding medium (organic matrix). The limits to the diffusion of ions are probably not due to any simple size factors, since - as will be reported in the next Chapter - the comparatively large tetracycline molecule can penetrate the whole bulk of "young" enamel quite rapidly.

3.5. <u>SUMMARY</u>. X-ray emission analysis showed that the concentration of calcium and hence the total mineral content increased approximately linearly with distance from the surface of the developing enamel. Calcification (mineralisation) does not occur in transverse relation to the incremental lines.

TETRACYCLINE LABELLING OF DEVELOPING RAT INCISOR ENAMEL

4.1.

The first experiments on the staining of enamel with vital dyes were those of BELCHIER (1736), DU MONCEAU (1739) and HUNTER (1770) who noted that pig enamel "takes no tinge from feeding with madder". MIZALDUS (1566 - cited by DU MONCEAU, 1739) was the first to notice that the "bones" could be stained in animals fed with madder. BLAKE (1799, 1801) found the teeth of rabbit foetuses tinged with madder, but was not sure whether the colour in the enamel was showing through from the dentine. LINDERER and LINDERER (1837 - cited by HENLE, 1841) found that enamel was coloured by madder. The latest experiments with the active principle of madder, alizarine, have been those of TONGE (1961) who found a retention of alizarine in the adult enamel of "intravitally stained rats."

The observation that the forming, soft enamel was coloured after the administration of certain vital dyes, and that the dye is lost from the "immature" enamel during "maturation" was made by GANZER (1906), who administered indigo-carmine subcutaneously to guinea pigs and studied the incisor enamel. The observation was repeated by WELLINGS (1915) using Trypan Blue in rats, mice and guinea pigs; MARSHALL (1920) using Dianil Blue RR in rats; and BLOTEVOGEL (1924) using Trypan Blue in the mouse.

GIES (1918) reported a retention of Trypan Blue in fully mineralised enamel after its administration to young rats, rabbits and dogs, but he did not prepare sections of the teeth and it is therefore possible that the colour was showing through from the underlying dentine.

The tetracycline antibiotics are selectively attached to the sites at which calcification is proceeding in bony tissues. The tetracyclines are not deposited in sufficient quantitites to give a visible colour to the calcified tissues, leastways, not a sufficient amount of colouration for microscopic purposes. They are made visible by their yellow flourescence when irradiated by ultra-violet radiation (MILCH, RALL and TOBIE, 1957,1958).

The potentialities of the method of staining developing, calcifying tissues with the tetracycline antiobiotics are very great; because of the widespread administration of these drugs to the human (MILCH, RALL and TOBIE, 1957, 1958; HARCOURT and JOHNSON, 1963; HARCOURT, 1963) and to domestic animal species (OWEN, 1961) in the normal, therapeutic course of events. The present investigations were commenced (Summer, 1960) before any reports of the tetracycline staining of dental tissues had appeared in the literature (OWEN, 1961). In a preliminary experiment (in collaboration with Dr. J.P. WATERHOUSE) two rats were injected with tetracyclines (Terramycin and Aureomycin) on three occasions at weekly intervals. The rats were killed after one more week; and transverse and longitudinal sections of their incisors prepared and examined under U-V irradiation (see 4.2.). The dentine calcifying at the time of injection was "labelled"; and the observation of SCHOUR et al (late 1930's) that the dentine is apposed at approximately 16μ per day in these teeth was confirmed. There was no observable staining of the enamel. These experiments were repeated in order to see if the tetracycline was ever bound to the enamel; that is, if enamel was stained, and then lost the stain again, as is the case with all other vital dyes.

4.2. Experimental Details Four 200 gram rats (Rattus norvegicus) were given 1.2 ml. of a solution containing 10 mg./ml. Tetracyn (Pfizer Ltd.) by intraperitoneal injection - they were killed (after 35, 78, 149 and 272 minutes) by decapitation. The heads were immediately dropped into an acetone/carbon-dioxide-ice freezing mixture (approximately - 75^{o}C) and then transferred and stored in the "deep-freeze"

4.3 (-10°C. approximately) compartment of a refrigerator at all times when the material was not being handled for sectioning. I undertook no experimental control of the effectiveness of this measure, which was aimed to prevent diffusion of the dye after death.

Longitudinal and transverse ground sections of the incisors were prepared (and stored in deep-freeze). The "young" incisor enamel was found to flouresce intensely under Ultra-violet irradiation

(It is normal to use quartz optical components in order to prevent absorption of U-V light. .In the present case it would have been desirable to have a non U-V absorbing condenser and microscope slides to achieve a good intensity of illumination at the specimen : these were not available. The specimens were irradiated with U-V light from above the microscope stage. Either no cover-slip , or a mica cover-slip was used to cover the sections. A yellow filter was incorporated in the microscope column to remove any stray U-V light.)

These preliminary observations were extended in order to attempt to establish (a) how quickly the Tetracyn entered the enamel (which might help to give an impression of the speed with which this substance might pass through the ameloblasts - or through the odontoblasts, dentine and enamel-dentine junction!): (b) how quickly the label was lost again:and (c) to relate the zones in which labelling occurred and was lost again to the zones of "maturation" in enamel assessed by other methods; e.g. retention of organic matrix in decalcified sections, mineral content assessed by microradiography (and polarisation microscopy?).

Twelve rats (weighing between 230 and 285 grams each) were given intraperitoneal injections of 1ml. of a 10 mg./ml. solution of Tetracyn; after 22½ hours a further intraperitoneal injection of 0.5 ml of the same solution; and after a further 72 hours another 0.25 ml. This final dose was administered intravenously (under ether anaesthesia) to three rats; which were killed at 80 secs., 2 mins. 20 secs., and 5 mins. respectively: and intraperitoneally to nine rats; which were killed after 10, 27 and 80 mins., 2, 4, 8, 12½, 24 and 96 hours respectively. The heads were frozen upon removal (as before) and ground sections of the upper incisors prepared later. The "young" incisor enamel in all these animals was found to be heavily "labelled" - but because previous injections had been given

4.4. it was not possible to determine how rapidly the tetracyn was taken up by the enamel. A further series of experiments was therefore conducted.

Eleven (330-465 gram) rats were given 1.25 ml. each of a 10 mg./ml. solution of Tetracyn. Three rats were given intracardiac injections and killed after 55 secs., 110 secs. and 5 mins. 33 secs. respectively. Eleven rats were given intraperitoneal injections and killed by decapitation (after 14, 22, 40 and 80 minutes; 3½, 7, 10, 24, 72 and 122 hours respectively); and the heads were immediately frozen as before. Longitudinal ground sections of the upper incisors were prepared for me later (by Messrs. HODGSON and LEWIN and Miss HUGHES, whose technical co-operation and assistance I gratefully acknowledge in context).

4.3. RESULTS. The "young" incisor enamel was found to flouresce bright yellow under U-V irradiation in the rat which had received an intracardiac injection 55 seconds before death and in the animal which had received an intraperitoneal injection 14 minutes before death. (Its absence from the enamel of the rat injected 110seconds before death may be been due to the injection not having entered the heart:it is obviously necessary to repeat these experiments using larger numbers of animals and with a more precise intracardiac injection technique where the short time-intervals are concerned). The dentine in these taeth was not "labelled".

The labelling was still prominent after 48 hours, but could no longer be detected in the incisor enamel of animals killed 5 or 7 days after the last injection of tetracyline.

4.4. DISCUSSION

4.4.1. Previous work. OWEN (1961) figured the flourescence (in the visible spectrum) of the "HUNTER-SHREGER bands" in incisors of dogs which had had tetracycline antiobiotics administered. HARCOURT and JOHNSON (1962) found that "enamel does not flouresce when viewed directly along the

4.5 ultra-violet beam" in tetracycline (intra-vitally) stained human teeth. OWEN (1961) and HARCOURT and JOHNSON (1962) do not make the significance of their observations clear. It is possible that the tetracycline molecule is adsorbed on to the surface of the apatite crystals in enamel in a particular orientation, and an unsuspected polarisation of the flourescent light may account for these effects. STOREY (1963) reported that young rat enamel is "labelled" by tetracycline antibiotics 15 minutes after administration (a result in good agreement with the present findings for intraperitoneal administration) but that the yellow flourescence under ultra-violet irradiation had faded in mature enamel; except in hypoplastic areas in the incisors, which were incidentally found to be more common in the tetracycline injected animals. HARCOURT (1963) found that the flourescent areas in human enamel were not fully mineralised. These results support the present observations that the tetracycline is lost from enamel during its maturation; or that it is altered and loses its ability to flouresce.

4.4.2. Present findings. The finding that the tetracycline had entered the mineralising incisor enamel within 55 seconds after intravascular administration, whereas mineralising dentine was not stained in the same tooth, but only in those removed twenty minutes after the administration of the drug, would suggest that the dye entered from the ameloblast side: since some"labelling" in the dentine would have been expected if the dye had passed through it.

The beautiful longitudinal ground sections which HODGSON et al prepared for me show an approximate correspondence between the zone of tetracycline "labelled" enamel and the amount of organic matrix which one might expect to retain after acid decalcification, ie. the young enamel" of CHASE (1935). HODGSON and LEWIN (1964, and unpublished work) are conducting further experiments in an attempt to relate the regions in which tetracycline staining occurs to the regions of different mineral content; and to the crystallite size in enamel and dentine.

It is generally accepted that labelling with tetracyclines occurs by the binding of these substances to the surface of the apatite crystallites in calcified tissues. It would therefore be expected that

4.6 the amount of tetracycline bound in a unit volume of tissue would be proportional to the surface area of the crystallites which it contains: this surface area increases as the diameter of the cyrstallite increases during the maturation of enamel. At a certain point, however, it must be assumed that the spaces between adjacent crystallites would be so reduced that they would no longer allow the diffusion of tetracycline molecules; or alternatively, that the crystal surfaces would no longer be available to the tetracycline molecule. Either mechanism would explain the absence or minimal amount of labelling in the more completely mineralised enamel. However, the loss of tetracycline from maturing enamel would also be expected to occur if it were bound to the organic phase (since this is drastically reduced in amount during maturation), or if it were unbound and just resided in the intercrystalline spaces (which are also reduced maturation).

A crystal whose surface was completely blocked by adsorbed tetracycline could (presumably) not grow. It seems fortunate then - from the point of view of the quality of enamel structure - that the binding to the surface of enamel crystallites does not seem to be permanent: the labelling is lost during maturation. Tetracycline is retained permanently by dentine; and also by bone, until it is resorbed. The reason why the labelling in dentine is permanent may be because the tetracycline is bound to the surface of crystals which are just reaching their maximum size in the normal course of events. The surface of completely formed dentine crystals is not available; perhaps because they are blocked by some element of the organic matrix. It has not been shown that newly formed dentine crystallites do not adsorb and lose the tetracycline label again, in the same way as do the enamel crystallites. The zone in which this process might occur and the time during which the growth of the crystals occurs are very much more limited in dentine: this might explain why it has not yet been possible to detect the effect - should it exist. It would be imagined that the already forming dentine crystallites complete their growth during the period after a single administration in which high plasma levels of tetracycline prevail. However, the loss of adsorbed tetracycline from the surface of enamel crystallites almost

4.7 certainly occurs well into the period in which the plasma tetracycline has dropped to an insignificant level. As evidence of this it may be noted that no trace of tetracycline labelling was found in the enamel one week after the last administration of the drug: the facts that the whole thickness of the incisor enamel is soft at one stage and that the estimated time taken to form the enamel layer at any one point is nine days (PINDBORG and WEINMANN, 1959) would indicate that - even in the rapidly developing enamel of the rat incisor - the enamel crystallites take much more than a week to complete their growth in diameter.

4.4.3. Vital Dye Experiments in Relation to Maturation

The questions of the origin of the mineral components deposited in forming enamel, and whether the ameloblasts play an active or passive role in the removal of organic components during maturation, must still be regarded as unsettled.

WASSERMANN (1941, 1943, 1944) held the opinion that the short ameloblasts associated with maturation are active in the removal of organic material. In 1944, he showed that the parenterally administered vital dye Trypan Blue was deposited in forming enamel and found in the formative ameloblasts, but not in the stratum intermedium at this stage. He found that the Trypan Blue was lost from the enamel but could be found in the stratum intermedium cells during maturation. He considered it "more probable that the short ameloblasts are taking the dye from the enamel matrix when the latter loses its color." He considered that "VON KOSSA" stained sections showed the calcium concentration on either side of the (young enamel)-(young dentine) junction to be equal, and that therefore, no calcium would pass the junction at this stage. (Von KOSSA staining depends on the presence of phosphate or carbonate!). He thought the situation was different during maturation, because there was no "afflux" of materials from the ameloblasts - and the odontoblasts had slowed-down dentine production - and that this rendered it very probable that the additional calcium salts deposited in the enamel during maturation reached it, via the dentine, from the pulp. He stated that "This is no mere assumption as there is evidence that the hard enamel as well as the maturing enamel is taking in a small amount of

4.8 calcium during life." WASSERMANN(1944) also considered that the layer of "cartilage cementum" (which is deposited on the surface of the enamel of guinea pig molars before maturation is complete) would effectively prevent the access of mineral ions to the enamel. WASSERMANN's "Histo-physiologic analysis" was, in my opinion, carried much too far and with too much confidence.

Other previous work related to direction of entry of mineral during maturation. WEINMANN (1943) administered single injections of strontium chloride to rats and observed that "disorganisation and degeneration of the ameloblasts" occurred. The ameloblasts eventually "recovered" but did not "resume matrix production and the hypoplastic enamel matrix (did) not undergo maturation". This, WEINMANN thought, "tends to show that not only matrix formation but also maturation.....is dependent on the normal function of the ameloblasts". However, he did not consider the possible effect of the strontium in blocking further crystal growth by an action on either the crystals or the organic matrix , and he did not attempt the experimental removal of the damagd ameloblast layer to see if maturation could continue in its entire absence.

LEFKOWITZ, BODECKER and SHAPIRO (1944) found that, after the experimental removal of the dentinal papilla from cat teeth, the enamel-organ degenerated into a squamous epithelium and that the young enamel which was already formed failed to mature. This evidence, they considered, showed that the dentinal papilla is necessary for maturation and that the enamel-organ is unnecessary. They took no account of the damage they caused to the enamel-organ. They also considered that the pattern of maturation reported by DIAMOND and WEINMANN (1940), i.e. in increments at right angles to the increments of deposition of the tissue, was evidence that the calcium salts were derived from the pulpal (inner) side of the enamel-dentine junction. In 1947, these authors, (LEFKOWITZ, SHAPIRO and BÖDECKER) reported that the already formed enamel proceded to complete maturation after the removal of the enamel-organ in dogs: this they considered as proof of the dentinal source of the enamel minerals. This is a false deduction - all that can be said is that an intact enamel organ is not necessary. It would be necessary to prevent access of minerals via the surface of the enamel to confirm their view.

4.9. GLASSTONE (1955) reported the calcification of isolated, developing enamel in vitro.

BELANGER (1957) established, by means of his microincineration studies, that the ameloblasts have a much greater mineral content (cellular content of fine ash) than the odontoblasts, during the maturation of rat enamel. This he considered as evidence that the enamel receives its minerals via the enamel organ before the eruption of the tooth.

REITH and COTTY (1961) placed zinc oxide fillings at different levels on the pulpal surface of the dentine of rat incisors, in order to prevent access of minerals to the enamel via the dentine. They found that the "pattern of enamel deposition was the same, regardless of where the filling was placed" and concluded, therefore, that calcium salts must enter the enamel via the enamel organ and not via the pulp.

4.5. SUMMARY - "Tetracyn" * administered intraperitoneally or intravenously to rats is very rapidly taken up by young enamel (via the ameloblasts) and then lost again during maturation. These results are discussed.

* PFIZER Ltd., Sandwich, Kent, ENGLAND.

CHAPTER V

ENAMEL STRUCTURE
(PERSONAL OBSERVATIONS ON FULLY-FORMED MAMMALIAN ENAMELS)

5.1. Introduction

This chapter contains the sections which describe my observations relating to the structure of fully formed mammalian enamels: these sections are either too short to be promoted to chapters in their own right, or they would repeat material which is included in this thesis in published form, but which needs further introduction or comment. Discussion of the experimental methods as such will be found in the relevant sections of this chapter:- the results, as far as they affect our comprehension of enamel structure, are discussed in chapters 6,7,8 and 9.

Chapter 2 dealt with observations on the structure of developing enamel: it seems unlikely that the pattern of crystallite orientation in adult enamel should change from the pattern found in the developing tissue. The only observations reported which dealt with the changes from the developing to the adult enamel, were those of the total mineral content (determined by scanning electron-probe x-ray emission microanalysis - Chapter 3) and an unknown factor concerned with the mineral content, i.e. its ability to "bind" tetracycline antibiotic molecules (Chapter 4). Further study of undemineralised sections of "mature" enamel by electron-microscopy and diffraction was not considered worthwhile, because of the severe distortion suffered by the tissue during "sectioning."

Note

I have made use of the very comprehensive collection of microscopic slides of ground sections of the teeth of mammals presented to the Odontological Museum (of the Royal College of Surgeons of England) by Sir Charles Tomes, to make good certain deficiencies in my own material, but in particular with respect to the members of the Order Cheiroptera. (Their enamel is of special interest in the following respects, viz:- (a) the prisms do not decussate, (b) they have Pattern 1 prisms, and (c) some members of the Order possess "enamel tubules".) I am grateful to the Curator of the Odontological Museum, Professor A.E.W. Miles, for permission to examine this material.

5.2. This Chapter serves to tie together various observations made on adult mammalian enamels using a variety of techniques. Some of these techniques have been applied for the first time to the study of enamel and their interest lies mainly in the future viz:- scanning electron probe x-ray microanalysis, scanning electron microscopy, ion-excited secondary-electron emission microscopy, physical erosion of the specimen surface under ion bombardment: my interest has been to weigh their applicability to the study of biological tissues - dental enamel in particular. The time spent using the instruments associated with the above techniques has been "borrowed" and I wish to make a general acknowledgement to the owners and users of these instruments for the generous facilities and co-operation which they have accorded me.

5.2. MATERIALS

The specimens examined by the various techniques considered in this Chapter have all been ground and polished sections or surfaces of mammalian teeth or natural or fractured surfaces of enamel. Ground sections were made of human, manatee, dolphin, horse, cow sheep, deer, pig, panther, cat, dog, kinkajou, rat, guinea-pig, coypu, capybara, squirrel, mole, hedgehog, shrew, elephant,(Nesodon - OWEN, 1846; order NOTOUNGULATA: suborder TOXODONTA - fossil ungulate) mastodon, kangaroo and opposum teeth. The sections were cut under a stream of water, using rubber and carborundum abrasive discs; at thicknesses varying from 1 mm. to 0.1 mm.: and finished on successively finer grades of metallurgical polishing (emery abrasive) papers (down to grade 4/0 - Oakey and Sons, Wellington Mills, London) to a final thickness of from 80-10μ (or approximately 0.5 mm. where only the surface of the sections was to be examined)..

In order to produce thin ground sections (less than 10μ) for light microscopy, the final stage of polishing was carried out with the specimen cemented to the glass microscope slide. A technique was worked out for the use of the special cement ("Lakeside 70") supplied for this purpose by the Cutrock Engineering Company Limited. This cement is normally used by heating it to 160°C., when it melts

5.3. and flows freely. In order to avoid "frying" the ground sections, a solution of this "Lakeside 70" cement in chloroform was prepared and used at room temperature. The cement had to be dried out in a 37°C oven for several days before the ground section was firmly attached to the slide. Later in this study, the method of FREMLIN, MATHIESON and HARDWICK (1961) which employs Kodak "Eastman 910" adhesive for the same purpose was used with far greater success: one of the great conveniences of this particular adhesive is the extreme rapidity (ca. 10 seconds) with which it sets.

The ground sections were either mounted in "D.P.X." or Canada Balsam for light microscopy, or stored in 70% alcohol. The smaller teeth were embedded in methyl methacrylate before sectioning, and these were either mounted directly - without removing the methacrylate - or stored dry.

5.3. Routine light microscopical findings.

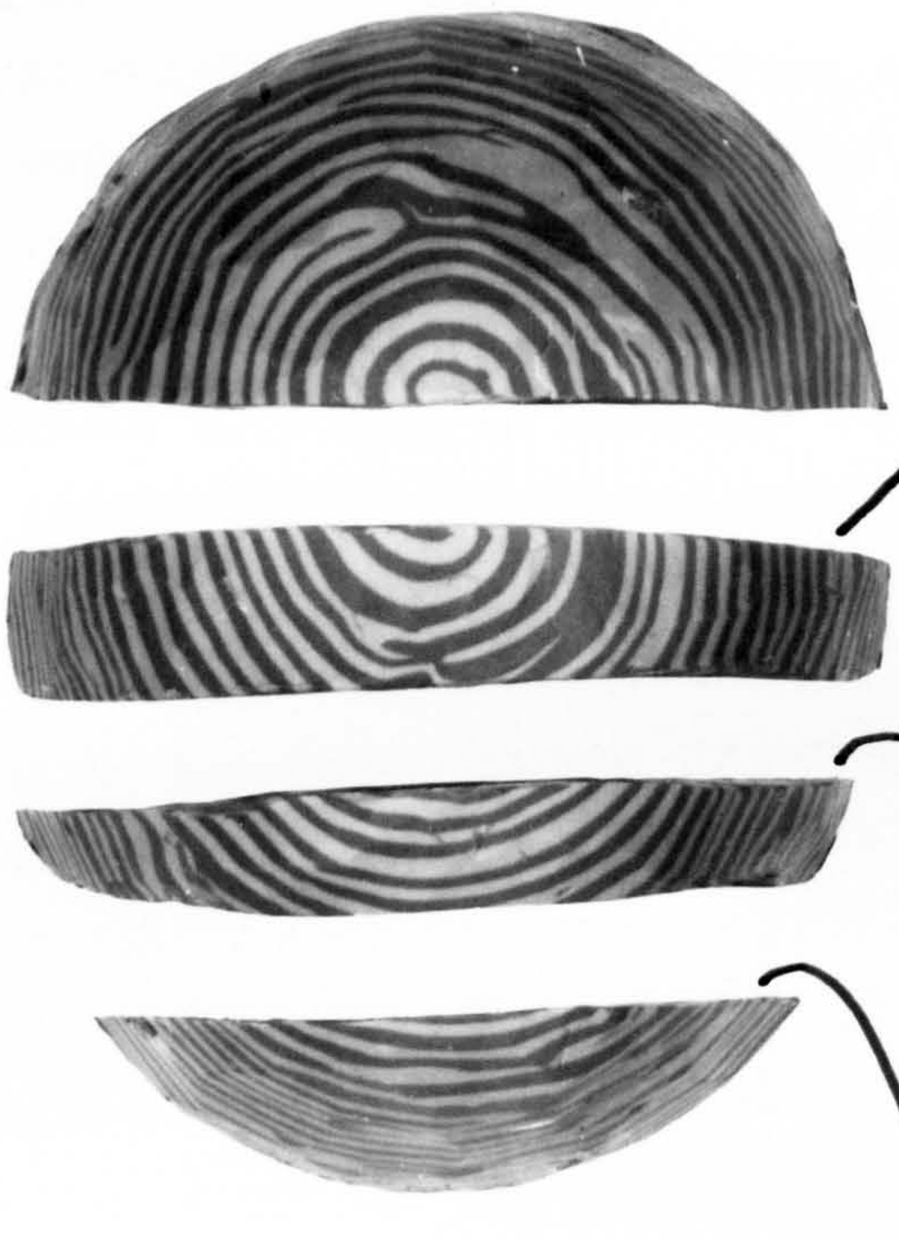
The Arrangement of the HUNTER-SCHREGER bands in human enamel. The earliest descriptions and figures of the HUNTER-SCHREGER bands in human enamel - as fibres or Striae by HAVERS (1689), de la HIRE (1699),BERDMORE, (1770), HUNTER (1770) and BLAKE (1798) - gave them as radially disposed from the surface of the dentine (Fig.5.1.). SCHREGER (1800) considered HUNTER'S (1770-1778) conception of their disposition (which seems to be the only earlier description of which he was aware) to be in error, in the respect that the "fasernstreifen" over the tips of cusps did not stand nearly perpendicular to the underlying dentine and did not all meet the dentine (Fig.5.2.). Recently STAZ (1946) and de BOER and STIEBELING have supported SCHREGER's view. De BOER and STIEBELING even considered that HUNTER may not have decribed the same architectural feature of the enamel but rather its cleavage directions("splijtrichting").

The examination of a number of longitudinal ground sections of human teeth showed that both types of arrangement of HUNTER-SCHREGER bands (zones) over cusps tips occurred. The arrangement described by SCHREGER (1800), STAZ (1946) and de BOER and STIEBELING (1959)

Figure 5.1 and Figure 5.2.
Photographs of sectioned Plasticine models of the arrangement of the HUNTER-SCHREGER bands over cusp tips. Zones of enamel in which the prisms run in the same direction are represented by the same colour Plasticine. Alternate zones contain prisms which run in different directions (the prisms decussate) and are represented by alternate colours.

The zone pattern begins as a spiral over the tip of the cusp. This is shown in the view from on top of the model on the left.

The appearance of the bands (zones) in longitudinal sections through the model cusp depends on how far the section lies from the centre of the cusp.
Top right. (Fig. 5.1). A section through the centre of the cusp shows the arrangement described by HUNTER with the bands radiating from and approximately normal to the surface of the dentine.
Middle right. A section to one side of the true centre of the cusp shows some of the bands separated from the surface of the dentine.
Lower right. (Fig. 5.2). A section even further from the midline of the cusp shows most of the bands "detached" from the enamel-dentine junction and possessing an almost horizontal disposition. This is the arrangement described by SCHREGER.



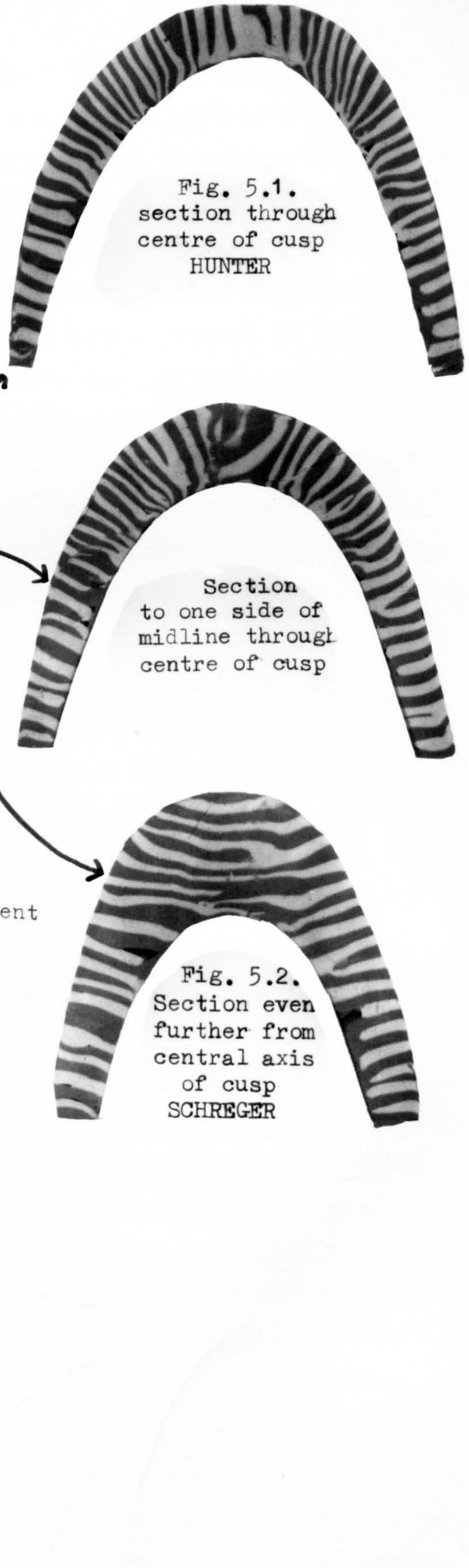


Fig. 5.1.
section through
centre of cusp
HUNTER

Section
to one side of
midline through
centre of cusp

Fig. 5.2.
Section even
further from
central axis
of cusp
SCHREGER

Figures 5.1 and 5.2. Photographs of sectioned Plasticine model of the arrangement of the HUNTER-SCHREGER bands. The pattern begins as a spiral over the cusp tip (view from on top). The appearance of the bands in longitudinal sections through the model cusp depends on how far the section lies from the centre of the cusp.

5.4. occurred more frequently than the true radial disposition, but the latter was present so often that it could only be considered as a normal finding. The degree of prominence of the arrangement considered normal by STAZ (1946) - i.e. the complete "detachment" of the cuspal HUNTER-SCHREGER bands from the dentine of the cusp tip - was increased with the degree that any given section diverged from being a true midline longitudinal section. In other words, the vertical radial disposition of the HUNTER-SCHREGER bands (zones) in this region is only seen in sections which pass through the centre of the tip of the cusp, and the SCHREGER-STAZ arrangement occurs in sections parallel to this, but further away from the cusp centre. The two apparently quite different arrangements of the HUNTER-SCHREGER bands in the cuspal enamel of human teeth have been described in the literature and both have been found in the present study. It therefore seems likely that both are manifestations of one and the same structural arrangement of the HUNTER-SCHREGER bands, and hence the prisms themselves. KAWAI (1953 p. 383, Fig.1A) figured the HUNTER-SCHREGER bands seen through the cuspal enamel (through the surface of the tooth from above; of a bear (Ursus-species not given) premolar:- the zone pattern can be seen to begin as a spiral over the cusp tip when viewed from above. This photograph suggested the idea of making plasticine models of the arrangement of the HUNTER-SCHREGER bands which could be sectioned in order to see if all the possible apparent configurations could be imitated.

Plasticine models were made in which the layers of alternately directed prisms were represented by different colours - and the dentine by a third colour (Figs. 5.1., 5.2.) The shape of the enamel was made up by wrapping a bi-coloured strip round itself, starting at the cusp tip and spiralling downwards. Care was taken to make the layers of the plasticine strip lie in the general direction of the prisms in the equivalent regions, that is, approximately normal to (and in contact with) the dentine surface. Such a model provides evidence that this might be the actual disposition of the HUNTER-SCHREGER bands in human and carnivore enamel. Looked at from above it shows a spiral dispositon: sectioned longitudinally through the centre of the cusp, the "bands" radiate from the dentine (Fig.5.1):

5.5 and when sectioned longitudinally to one side of the centre of the cusp(Fig.5.2.) the bands do not meet the dentine but show the configuration described by STAZ (1946).

(PICKERILL (1913) and WILLIAMS (1923 A) considered the course of the prisms to be spiral - and this to be responsible for the appearance of the HUNTER-SCHREGER bands - they did not describe the spiral arrangement of the zones themselves).

The teeth in museum skulls of various mammals were examined directly using a low-powered (x25) binocular microscope. The HUNTER-SCHREGER bands could be seen in the depth of the enamel in all the carnivores examined: which included 5 seals (Phoca vitulina - Carnivora Pinnipedia); 2 dogs and 1 wolf (Canidae); 3 large cats (Felidae- various unidentified species); 2 badgers(Meles meles); and one bear (Ursus - unidentified species). The zone-pattern could be seen to begin as a spiral over the tips of the majority of the cusps which possessed a form which was sufficiently blunt to permit examination (and were not damaged by attrition or fracture). It was particularly noted that the HUNTER-SCHREGER bands were disposed as concentric lamellae about the centre of even the smallest cuspules; and that this proved to be a simple way of defining the centre (and the existence of)"cusps": the palatal cingulum region of the large upper molars of the badger (Meles meles) and mink (Mustela: Fam. Mustelidae) show a number of small elevations, and each one of these has its own spiral (or circular) zone pattern.

The most prominent cusps of contralateral pairs of teeth were examined in the bear, wolf, "cats" and dogs in order to see if the zone pattern spiral began in any particular direction: the spiral began in opposite directions (clockwise:anticlockwise) in 18 out of 28 pairs; in the same direction in 4 pairs and the spiral arrangement could not be detected (i.e. the zone pattern began as a series of concentric circles?) in at least one member of each of the remaining 6 pairs. Of the 18 "opposite-handed" pairs,12 were from the upper jaw; 8 of these began as anticlockwise (from the centre outwards: viewed from the outside of the tooth) and 4 as clockwise spirals in the left quadrant. Of the 6 pairs from lower jaws, 4 left teeth showed anticlockwise (and 2 left teeth clockwise) spirals. There was thus

5.6 a 2:1 ratio in favour of the anticlockwise spiral in teeth of the left side (clockwise on the right side) amongst the "opposite handed pairs". This result would need to have been based on a much greater number of observations before it could be held to be significant: however, it does suggest that it would be worthwhile looking for the correlation.

The Ursidae would be the ideal group in which to further this study, since the spiral pattern is most easily distinguished in the low cusps - particularly of the unworn premolars - of these mammals. However, the more general availability of dogs, and the fact that their premolars usually remain essentially unworn, would make the choice of this species a matter of convenience.

Comparative anatomical observations of the cross-sectional outline of enamel prism boundaries: The technique used by SÜSS (1940), BLECHSCHMIDT (1942) and SHOBUSAWA (1952) to reveal the "prism-sheaths" was employed. Facets were polished on the "lateral enamel" of whole teeth so that regions in which the prisms were "sectioned" approximately transversely were exposed. The teeth then etched with NHCl for 20 seconds; washed;and stained by immersion in Ehrlich's Haematoxylin for approximately 2 hours. Surplus dye was removed by polishing the surface on a soft cloth. The surface to be examined was covered with a film of cedarwood oil and examined directly by reflected light. Prism boundaries showed as clearly defined dark purple lines. The resolution possible with this method is better than can be obtained during the transmitted light examination of ground sections of the same material, since the image "information" comes from a relatively thin layer in the specimen surface.

PATTERN 1 prisms (Fig. 1.1 - "circular" prism-sheaths separated by interprismatic regions) were the most commonly found in Globiocephala, Phocaena and Tursiops (Odontoceti); Erinaceus europaeus (Insectivora); and Trichecus latirostris (Sirenia).

PATTERN 2 prisms (Fig. 1.2. - longitudinal rows of prisms with interrow sheets of interprismatic material) were found Bos bovis (Ungulata)and Didelphis nudicaudata and Macropus (Marsupialia). A modified (by the prism decussation) PATTERN 2 was found in Cavia cobaya and Myocastor coypus incisor enamel (Rodentia).

5.7 PATTERN 3 prisms (Fig. 1.3. - arcade or fish scale arrangements) were found in Phoca vitulina (Carnivora Pinnipedia)- the kinkajou (Potos; Carnivora Fissipedia) and in human and Rhesus macacus (Primates) enamels (PATTERN 1 and PATTERN 2 -prisms were also found in human and monkey enamel).

These results, which show that there is a marked degree of preference of particular prism patterns in particular mammalian orders, confirm those obtained from the examination of electron-micrographs of ultra-thin sections of developing enamel; (see Chapter 2) ; and are in agreement in all essentials with those of SHOBUSAWA (1952: which are detailed in the next chapter - Chapter 6).

5.4. Estimation of the age at death of young human skeletal remains from incremental lines in the enamel (see appended publication).

The evidence that the cross striations of human enamel prisms are daily increments of growth (ASPER, 1916; KOMAI, 1942) was accepted: the total number of cross striations from the neonatal line (RUSHTON, 1933, SCHOUR, 1936) to the last formed enamel was used as an estimate of the age (developmental) in days. This method is of interest for forensic and archaeological investigations.

5.5 Replica techniques for electron microscopy.

Metal shadowed formvar replicas and platinum-carbon replicas of ion-etched section-surfaces (in particular - see section 5.8 and BOYDE and STEWART, 1962), polished and acid-etched surfaces, fractured or natural enamel surfaces were examined by transmission electron microscopy ... Formvar replicas were made using a 2% solution in chloroform which was flooded on to the specimen surface, the surplus poured off; and the remainder allowed to dry. The formvar films were reinforced with a "Sellotape" backing strip before being stripped off as replicas. The evaporation of metal (shadowing) on to the replica surface was usually conducted before the removal of the replica from the "Sellotape" backing. The replicas were dissected with a very sharp blade, whilst still attached to the "Sellotape", thus selecting grid-sized areas (i.e. approximately 2.3 mm. in diameter) for examination in the Siemens Elmiskop I electron microscope: the replica material was floated off from the "Sellotape" in petroleum ether, and collected and mounted by "fishing" it out with an E/M grid.

5.8 The carbon or platinum-carbon replicas were prepared via the use of "Triafol" replica material. The specimen surface was wetted with acetone, and the "Triafol" sheet material pressed on with the thumb. The acetone dissolved the "Triafol" sufficiently for it to adapt to the irregularities of the specimen surface. The replicas were stripped after a short drying period (1-10 mins) and covered with a layer of evaporated carbon or platinum-carbon. The area of interest of the replica was then cut out with a sharp blade; and an E/M grid placed over it; i.e. on to the replica surface, now covered with carbon. The grid was sealed on to the replica with a drop of molten wax applied through the back of the grid. The "Triafol" replica material was dissolved away during some 30 minutes in a bath of methyl acetate saturated in the same(histological paraffin) wax. Finally, the wax was removed by placing the grids, wax-side downwards, on a filter paper "bridge" over which a very slow stream of chloroform was allowed to run from a burette.

Replicas were also prepared via the water soluble medium poly-vinyl alcohol. This proved to be a very reliable method, in the sense that the replicas could always be stripped complete. However, a long drying period was required if a sufficient amount of replica solution had been applied for a tough replica to result. These replicas were very suitable for light microscopy after aluminium shadowing and mounting in Balsam. For electron microscopy, the poly-vinyl alcohol replicas were covered with a layer of evaporated carbon; and the poly-vinyl alcohol dissolved away in water. The latter proved to be a very slow, unreliable and exasperating process. The very few successful (two-stage carbon)replicas prepared by this method were collected directly on to E/M grids.

5.6. SCANNING ELECTRON MICROSCOPY

5.6.1. Introduction: Materials and Methods

The basis of this method has been touched on in Chapter 2. and is treated more fully in papers bound at the end of the thesis - BOYDE, SWITSUR and FEARNHEAD (1961), BOYDE and STEWART (1962) - and in the references appended to these papers.

Figures 5.3., 5.4., and 5.5.
Scanning electron micrographs (Cambridge Instrument Company "Stereoscan" development model) of various surfaces covered with a layer of gold-palladium by evaporation in vacuum.

Fig. 5.3. (top left) Fractured enamel surface. The field shows a region in the centre of the enamel on the buccal face of a human lower first permanent molar (longitudinal bucco-lingual fracture). (X 1300).

Fig. 5.4a. (top right) Low power view (X 130) of a fragment of Palaeomastodon molar enamel surrounded by Araldite (upper left half of the field) after acid etching. This image shows nicely the depth of focus of the microscope and its easy "scanning" facility (the term scanning in the name of the microscope refers to the raster scanned by the electron beam on the specimen surface).

Fig. 5.4b (centre left) The same specimen (X 400). The field shows a zone of longitudinally "sectioned" prisms with clearly marked cross-striations.

Fig. 5.4c. (centre right) The same specimen again (X 1300). A field of transversely "sectioned" Pattern 3 prisms.

Fig. 5.5a. (lower left) The natural surface (after scrubbing, drying, and "metallising") of a human lower first permanent molar near its mesial contact area. The broken edge of the specimen can be seen at the far left hand side of the field; three Perikymata run vertically and two scratches obliquely (from WNW to ESE) across the field. (X 130)

Fig. 5.5b. (lower right) The same specimen (X 1300) The absence of clearly defined "impressions of the enamel rod ends" was surprising.

The above pictures are photographs of Polaroid-Land photographs of the cathode ray display tube of the S.E/M. There has been an inevitable loss of fine tone differentiation in these successive reproduction stages.

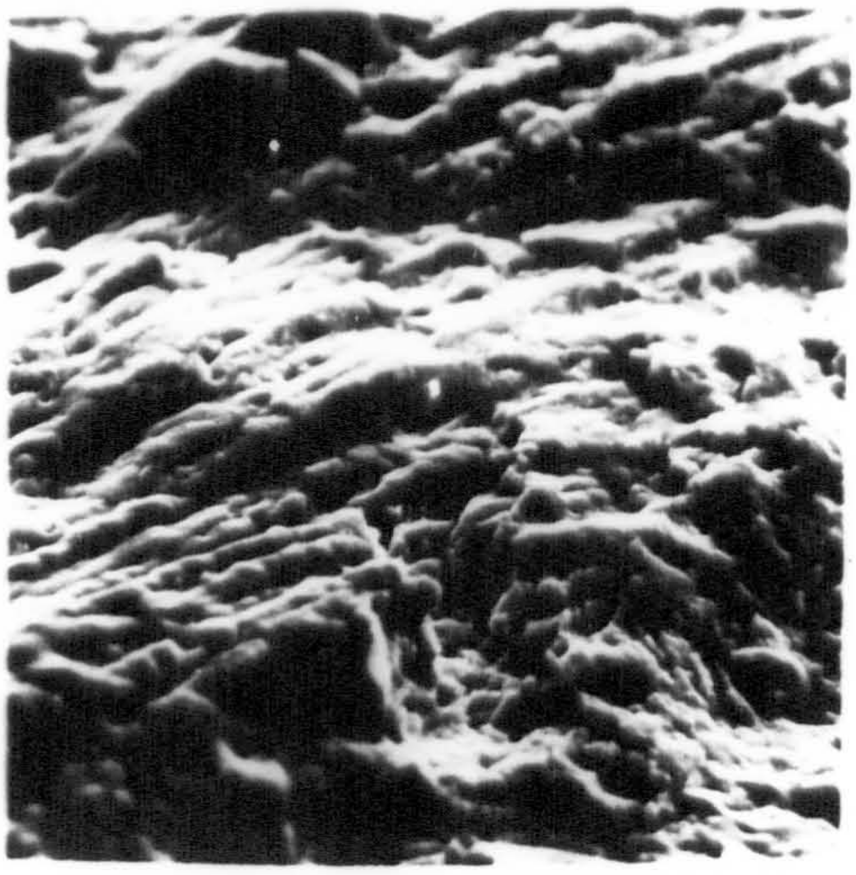
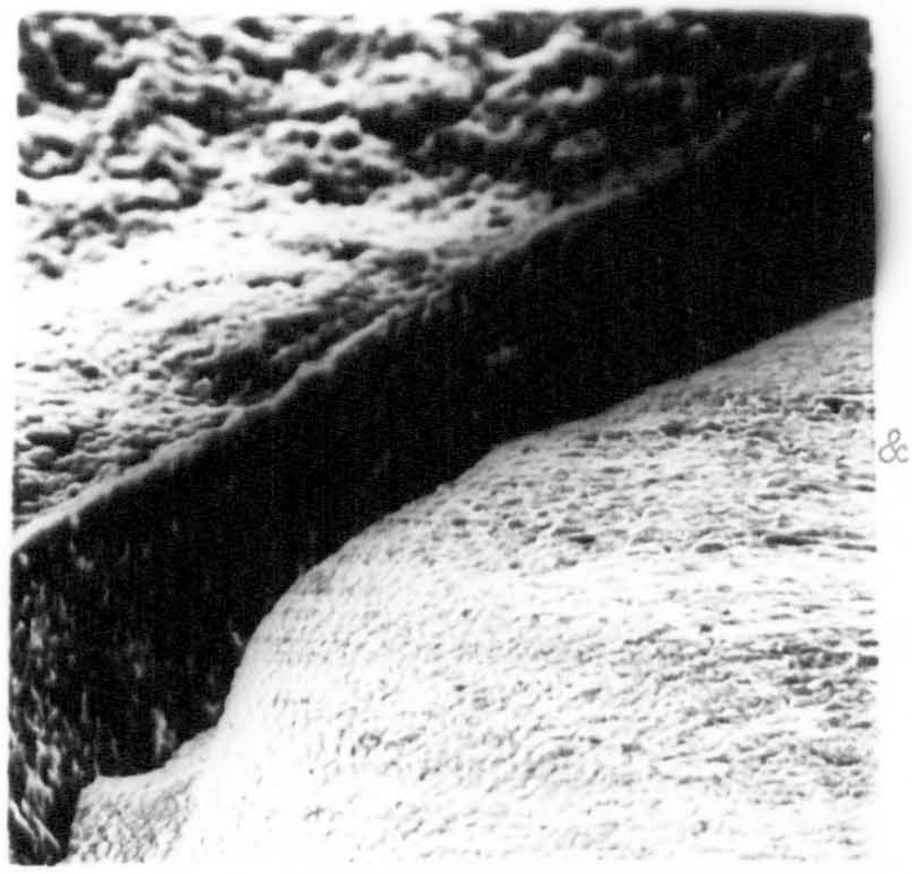


Fig. 5.3. Fractured human enamel. (X 1300)



Figs. 5.3. 5.4. & 5.5

Fig. 5.4a. Acid etched Palaeomastoden enamel. (Araldite above, X 130)

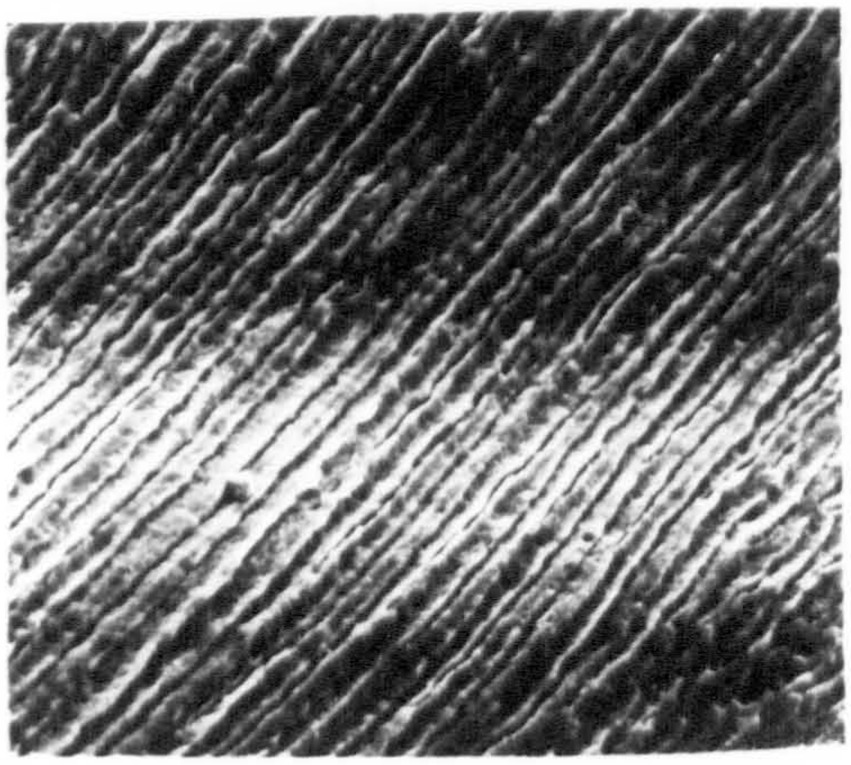


Fig. 5.4b (same, X 400)

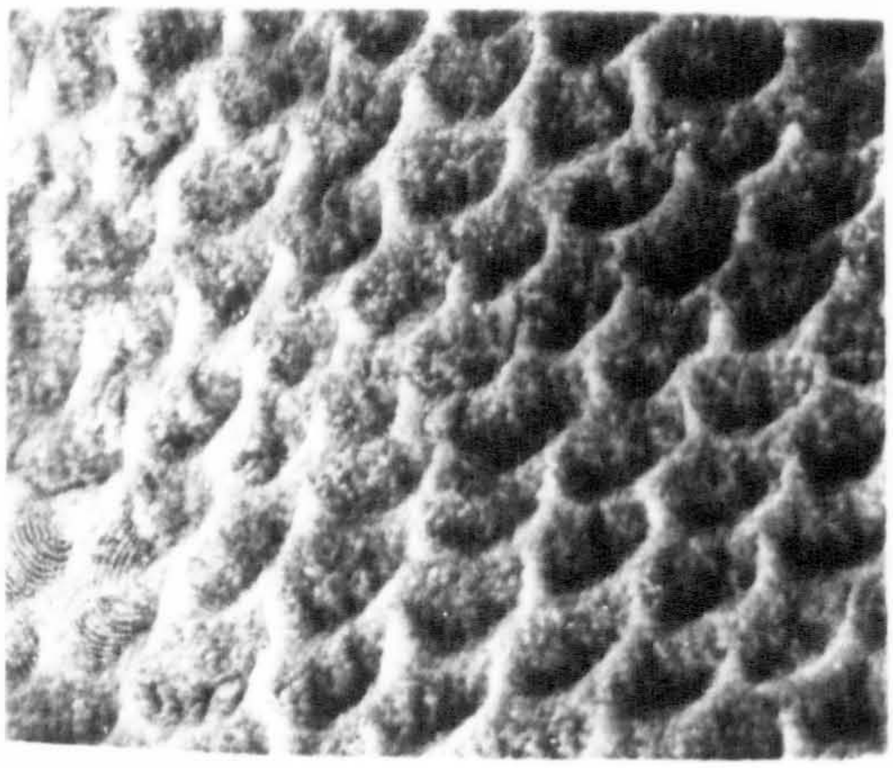


Fig. 5.4c (same, X 1300)

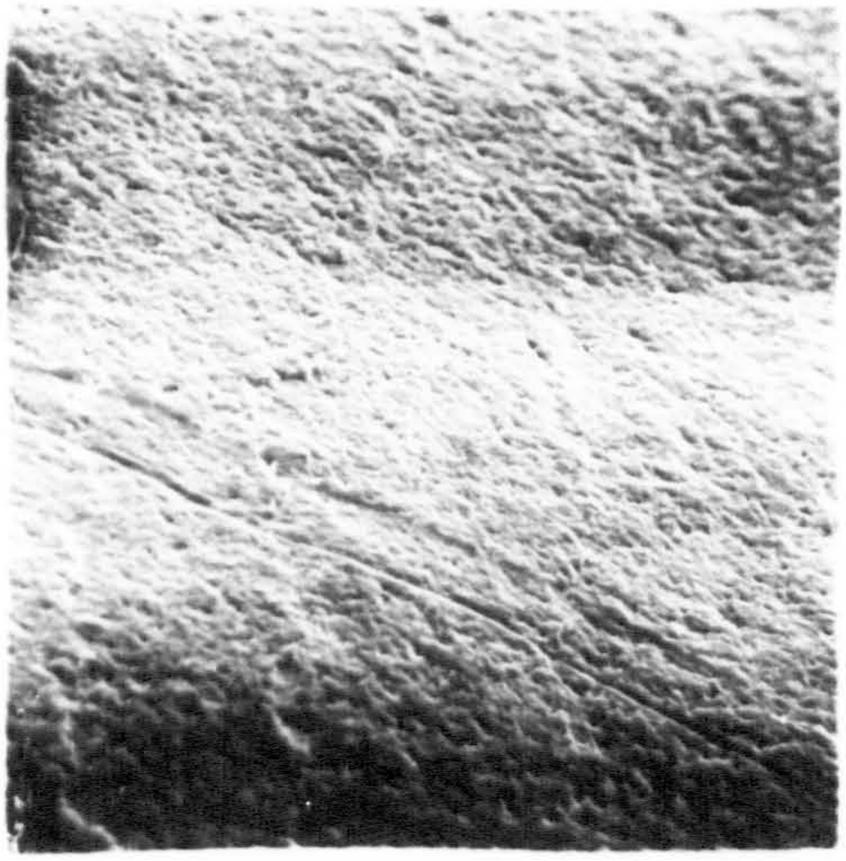


Fig. 5.5a Human: tooth surface. (X 130).

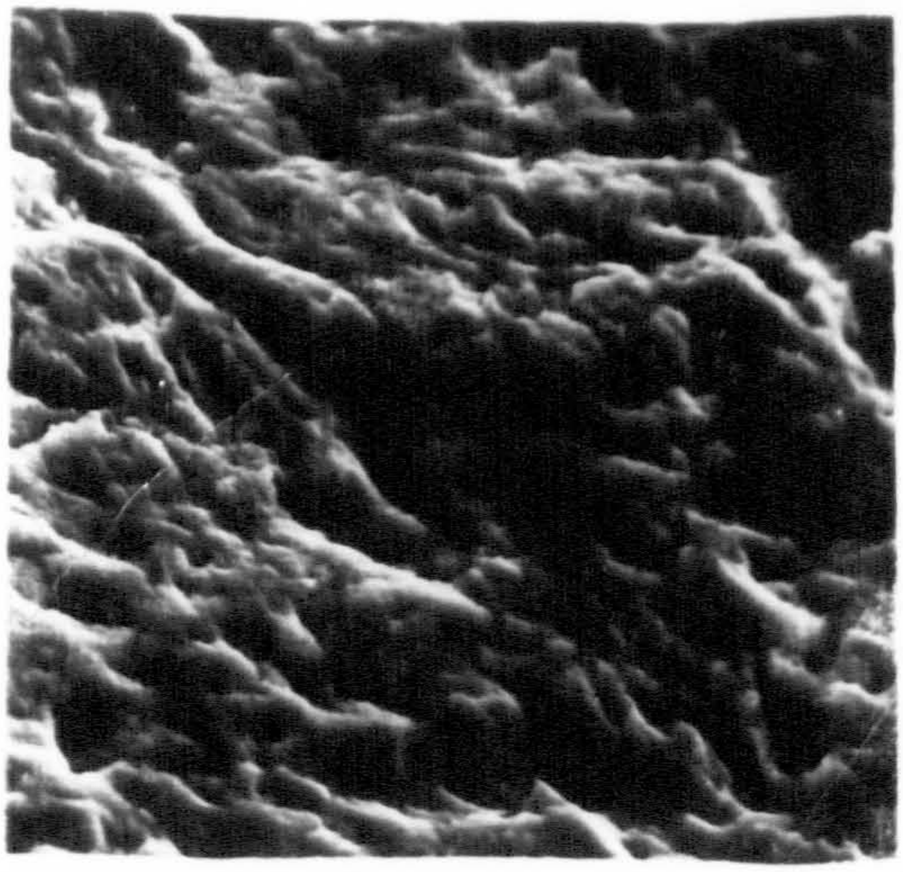


Fig. 5.5b (same, X 1300)

5.9. The surfaces of polished and acid-etched sections or fragments and fractured and natural enamel surfaces were studied using the Cambridge Instrument Company developmental "Stereoscan" instrument. These are reported here for the first time. The specimens were portions of adult teeth. The details of preparation of the specimens for the Cambridge Instrument Co. SE/M have been given in Chapter 2. and for STEWART'S SE/M with attached ion source in BOYDE and STEWART (1962).

Natural tooth surfaces. Specimens of human mandibular first permanent molars were prepared so that the approximal (contact area) surfaces were exposed to view (Fig. 5.5).

Fractured surfaces. Specimens were prepared such that the surfaces of longitudinal fractures of the enamel were exposed to view (Fig. 5.3.).

Acid etched surfaces. A small fragment of enamel from a (fossil) Palæomastodon molar was heavily acid-etched after mounting on the specimen holder (Fig.5.4.).

Conducting films. The specimen surfaces examined in the "Stereoscan" were covered with a layer of evaporated Gold-palladium alloy or palladium some 100 Å thick. Aluminium conducting films were applied to the specimens examined in the other scanning electron microscopes used, i.e. STEWART's at the Engineering Lab., University of Cambridge; the Cavendish Microanalysers (V.R. SWITSUR); and the Cambridge Instrument Company x-ray microanalyser ("Microscan").

Instrument operation. Accelerating voltages of from 12-16 kV (25 kV -"Cavendish" Microanalysers) were employed and the images were formed using either the high energy reflected electrons, or the low energy secondary electrons emitted from the specimen surface under bombardment.

5.6.2. RESULTS: Discussion and Conclusions

There can be little doubt that scanning electron probe reflection microscopy has any important role to play in future advances in enamel histology, and particularly in the study of natural enamel surfaces. The facility of being able to examine large areas of a very simply prepared specimen by "scanning" it at a low magnification

Fig. 5.6.

Figure 5.6. Photograph illustrating plane of section of ground section (Q) of human upper premolar (male, age 25), the study of which by argon ion beam etching-erosion in a scanning electron microscope was reported on by BOYDE and STEWART (1962).

The specimen after mounting and aluminising.

5.10 (e.g. x100) and then switching up to a high magnification (e.g. x 50,000) when an area of interest is located has considerable appeal.

We have not yet applied this technique extensively enough to be able to report any new findings. However, it seemed that some of the histological features which were expected to be found easily in the scanning electron image were conspicuous by their absence, e.g. the "enamel-rod ends" on the true enamel surface. Fig. 5.5 shows the typical appearance of the enamel surface in scanning electron micrographs. Perikymata could be seen at low magnifications (Fig. 5.5a).

5.7. Scanning Electron Probe X-ray Emission Microanalysis.

Details of the method, specimens and operative conditions used are contained in papers appended at the end of this thesis (BOYDE, SWITSUR and FEARNHEAD, 1961; BOYDE, SWITSUR and STEWART, 1962; and BOYDE and SWITSUR, 1963; SWITSUR and BOYDE, 1963).

BOYDE, SWITSUR and FEARNHEAD (1961) provided the first quantitative analysis of the amount of iron in the pigmented layer of a rodent (Rattus norvegicus) incisor. We have also found a sizeable proportion (ca. 5-10 %) of iron in the surface enamel of shrew molars and coypu and squirrel incisors (BOYDE and SWITSUR - unpublished).

The CaK_α pen trace figured in the publication by BOYDE, SWITSUR and FEARNHEAD (1961 p. 204, Fig. 2b) shows a lower mineral content in the middle layer of the enamel, a slightly raised concentration towards the enamel-dentine junction, and a yet more raised level towards the enamel surface, just inside the iron pigmented zone.

5.8. Argon Ion-Beam Erosion and Etching of Polished Enamel Surfaces in a Scanning Electron Microscope.

The surfaces of tooth sections were bombarded with 5 keV A^+ ions in a vacuum of 10^{-6} mm.Hg. The specimen surfaces were examined before and after ion-bombardment, both in the scanning electron microscope in which the bombardment took place; and by replica techniques (Section 5.3.2.) for light and electron microscopy. Full details of the techniques and the instrument used

Figure 5.7. (top) Transmission electron micrograph (Siemens Elmiskop I, X 2410) of Palladium shadowed Formvar replica of a section of Macropus molar enamel etched with a 5keV Ar^+ ion beam (in a scanning electron microscope), showing projections (the very electron dense, short, thick lines which were pulled out of the open ends of the enamel tubules. The large white area at top right is a hole in the replica film. The exposure was made with the specimen, i.e. the replica mounted on an E/M grid, tilted on a special stage in the E/M so that only the central parts of the image are in focus. The area of enamel shown lay just inside the enamel from the enamel-dentine junction, very close to the region shown in the scanning electron micrograph figure 5.8.: the density of enamel tubules is greatest in this region. Notice that the (replicas of the) "tubules" all arise from the ends of pits in the surface.

(bottom) Two transmission electron micrograph images (X 4850) of a similar field recorded with the specimen having different tilts with respect to the electron beam: the two images constitute a stereoscopic pair .The two images should be examined with a special "Stereo viewer", when it will be seen that they have been arranged such that the side of the replica stripped from the specimen surface is uppermost: the projections from the surface (i.e. the replicas of the tubules) arise from the top of mounds in the plane of the replica. Reversing this picture in the mind will show that the tubules are related to the bottom of ion-etched pits. (Surface after $70\frac{1}{2}$ mins. ion etching).

Figure 5.8. Scanning electron micrograph (X 2000 in the axis WSW to ENE, X 1450 in the axis NNW to SSE: the specimen was tilted at 45° to the electron beam) of ion etched section of Macropus molar showing the dentine (below) eroded more deeply than the enamel (above) so that a cliff has formed at the enamel-dentine junction. Note the pits in the enamel (to the bottom of which the enamel tubules are related - see Fig. 5.7) and that the dentinal tubules run right up to the enamel-dentine cliff. (Two intersecting cracks form the dark cross in this image). ($40\frac{1}{2}$ mins. etch.)

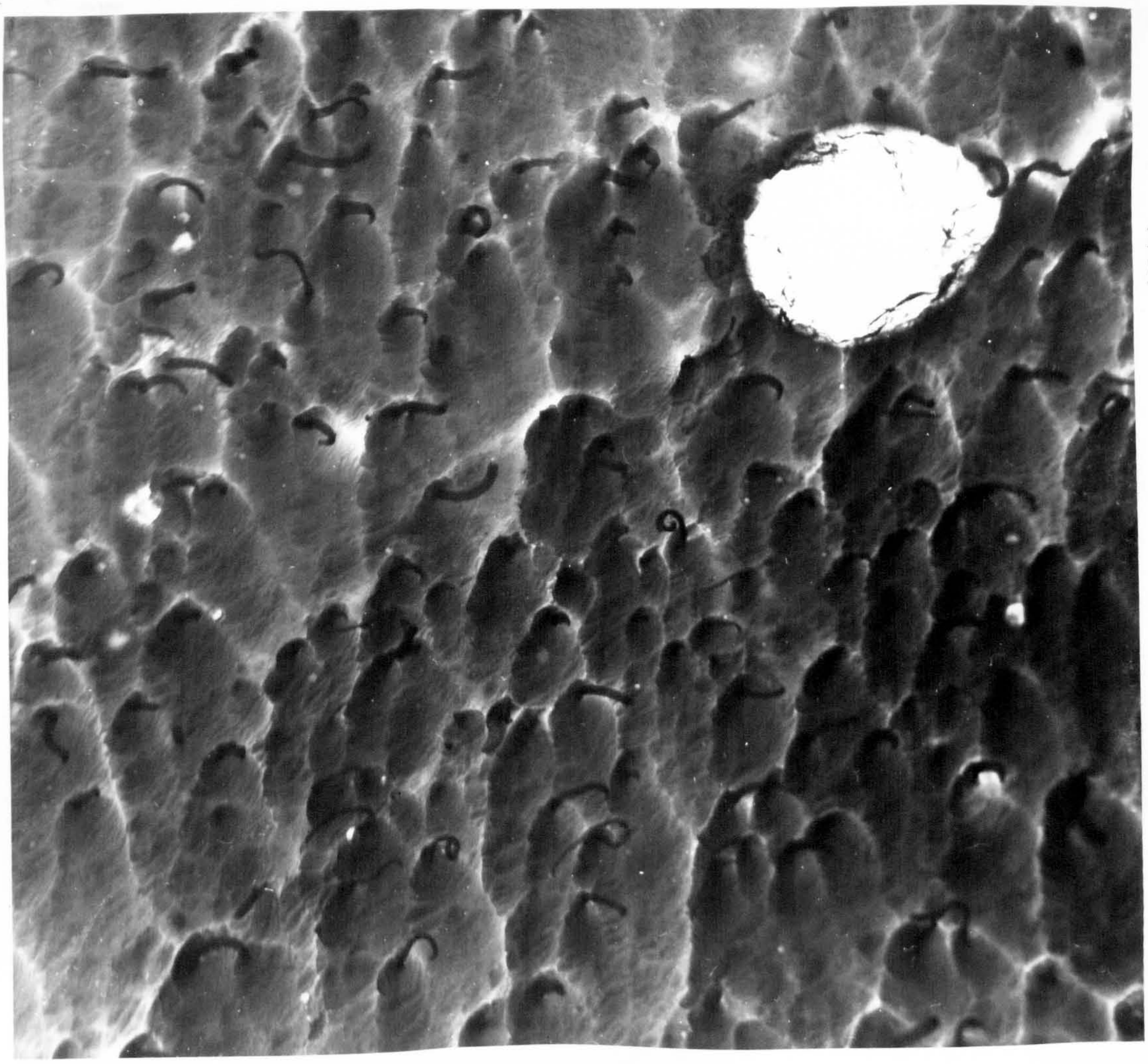
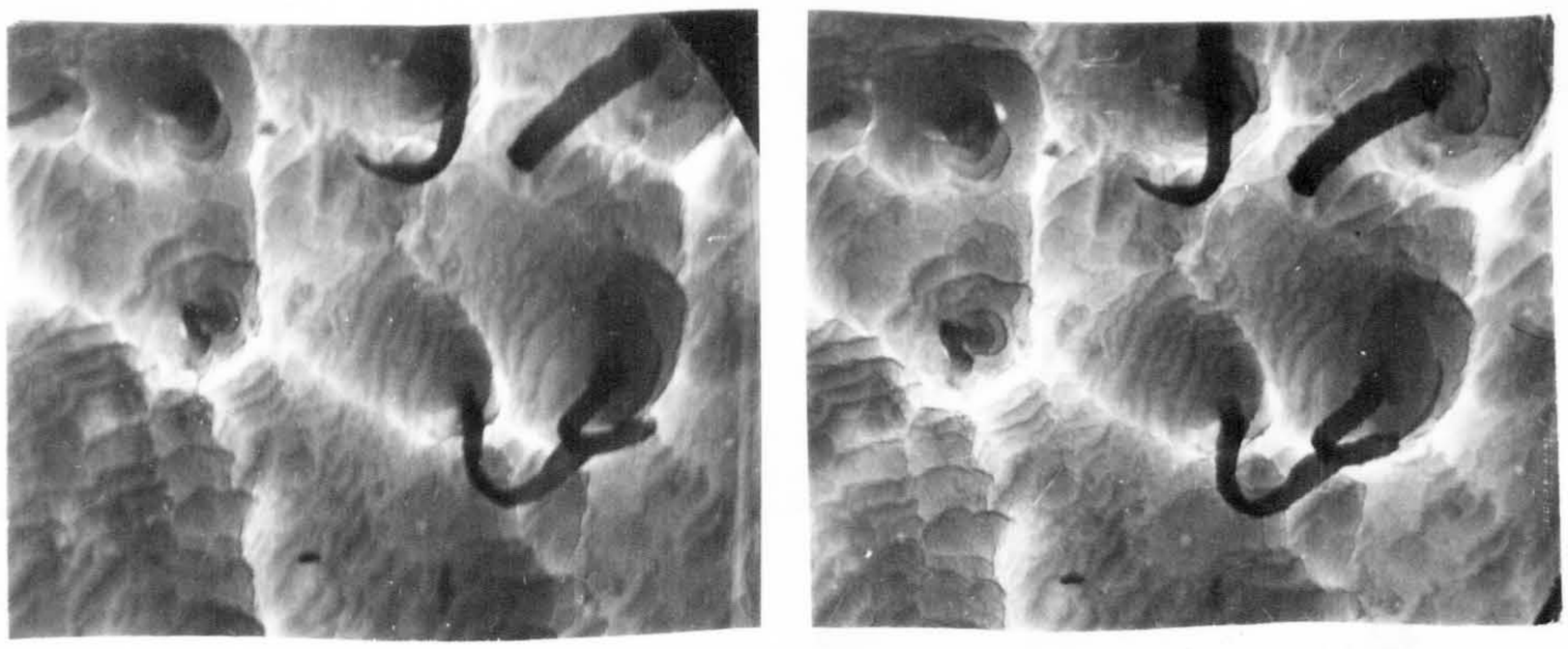


Fig. 5.7.

Figure 5.7. Macropus: Transmission electron micrographs of replicas of ion-etched enamel. (above, X 2410)



(Stereo pair, X 4850)

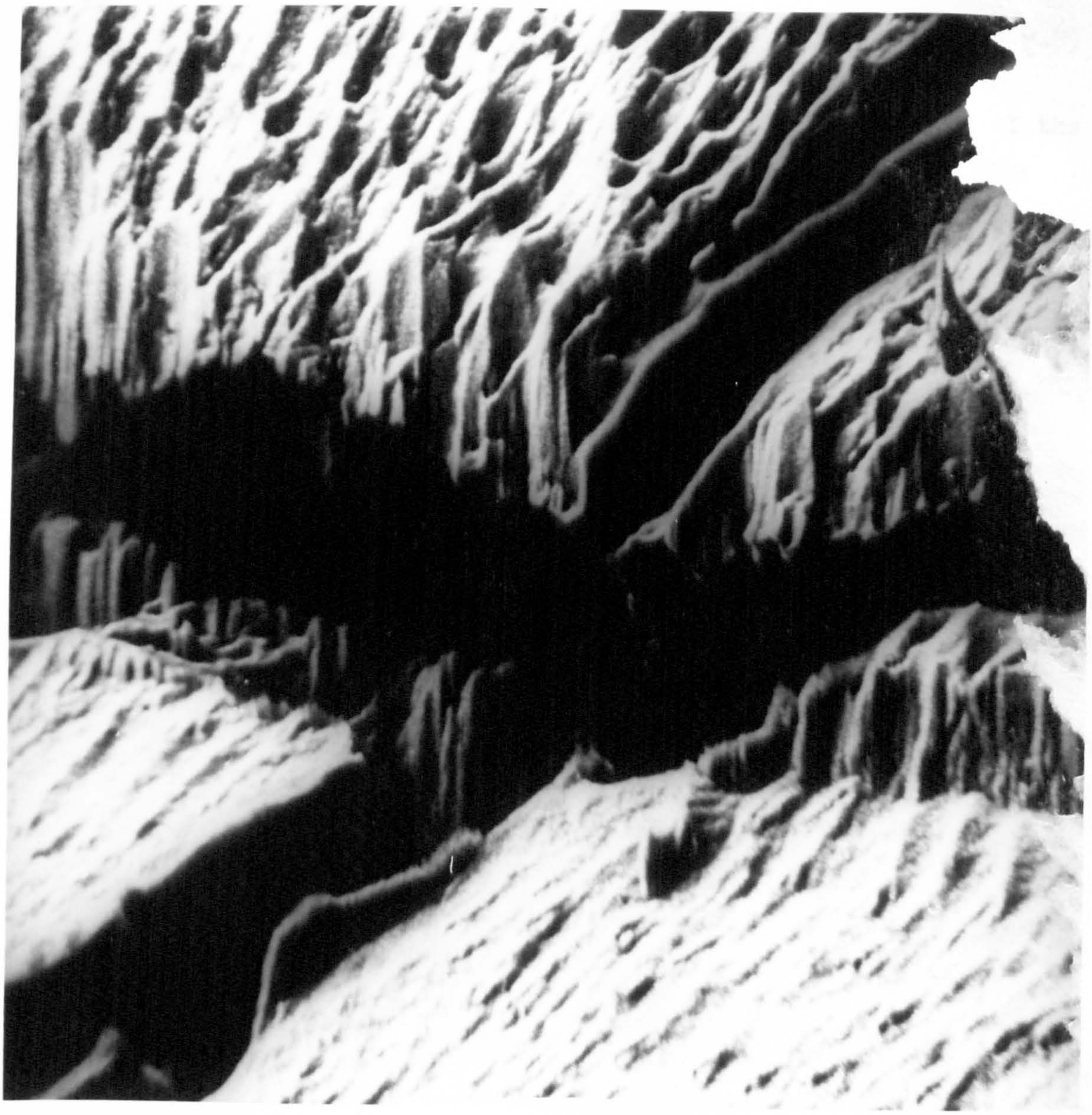


Figure 5.8. Macropus: scanning electron micrograph of ion-etched enamel (above) and dentine (below). (X 2000)

5.11 are given in STEWART and BOYDE (1962), BOYDE and STEWART (1962 A,B) and BOYDE, SWITSUR and STEWART (1962).

Fig. 5,6 shows the details relevant to the preparation of the specimen considered at length in BOYDE and STEWART (1962). The following comments (1, 2, 3) might be added to the end of this paper.

1) The Figure 6a (p.169) shows a tuft region having a width of some 15-20μ. The orientation of the section-surface (as can be seen from Figure 5.6) was such that this would be a real impression of the width of the tuft, i.e. the tuft is not sectioned so obliquely that its apparent width would have been significantly increased. A number of tufts were studied in this specimen and they were all in this range of width; that is, they were by no means confined to the plane of interprismatic substance between two adjacent fields of prisms - a disposition which has been suggested by some authors (see section 9.3).

2) Many replicas were obtained from the surface of the argon-ion etched Macropus molar enamel (mentioned in BOYDE and STEWART, 1962; Figure 7, p.169). Long extensions of the replica material (formvar) were pulled out of the enamel tubules (Fig. 5.7). Ion-etching seems to have been a particularly suitable way of preparing the enamel surface for this purpose. (No success was achieved in attempts to "replicate" the enamel tubules in fractured, or acid-etched marsupial enamel surfaces). The "tubules" always arose from the bottom of an ion-etched pit.* This is why they cannot be seen (in the shadow of these pits) in the scanning electron micrographs of the same surface (Fig.5.8). Figure 5.8. shows the steep cliff formed as a result of the different sputtering rates of the two tissues at the enamel-dentine junction in Macropus. The dentine tubules can be seen to run right up to the enamel-dentine junction.

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* STEWART (Ph.D. Thesis Cambridge - in preparation) provides theoretical considerations related to this observation. The effect may be due to a (highly localised) focussing of ions into the openings of transversely sectioned enamel tubules; thereby leading to a localised increased rate of bombardment and sputtering: or to the tubular contents (and their immediate surroundings?) being of different composition and therefore sputtered away more rapidly. However, it is still necessary to explain how the sputtered material can escape from the specimen if it is ejected into the tubular lumen?

3) <u>BEILBY layer on polished enamel surfaces</u>. Differential etching of ion bombarded "polished" enamel surfaces did not commence until a few microns of the surface enamel had been eroded away. (It must be assumed that approximately $\frac{1}{2}$-1μ of surface material would need to have been removed before potential differences in the rate of erosion of differently constituted or oriented zones would be revealed: that there was a delay in the onset of differential erosion in the immediate surface layers of polished enamel surfaces was judged in comparison with the rate at which this developed in the sub-surface layers. A much more marked delay in the onset of differential erosion occurred in the depth of reference scratches applied to the (enamel) specimen surfaces). It was deduced from this that polishing (or scratching, i.e. applying a large load to a small area) can produce a BEILBY (damaged, amorphous) layer on enamel (BOYDE and SWITSUR, 1963). The existence of such a layer has also been noted by FOSDICK, McMILLAN and BLACKWELL (1959), BERLIN (1959) and SCHMIDT (1961). The thickness of this BEILBY layer of plastic deformation cannot be deduced from the present results. It might be measured in replicas of surfaces fractured perpendicular to polished surface (the crystallite orientation pattern cannot be seen in the BEILBY layer - BERLIN, 1959) or by a highly refined selected area reflected electron diffraction technique (using a finely focussed e.g. 500 Å diameter - electron probe: arced patterns would be obtained in the undamaged sub-surface layers). The existence of the damaged layer is obviously disadvantageous where a physical technique used to a examine a specimen is based on "information" obtained from its surface layers; e.g. in scanning electron probe x-ray microanalysis.

Experiments with methods of surface preparation which might not produce deformation are in progress: for example, using "airbrasive" finishing and ion beam machining (BOYDE - unpublished) and using a very high velocity acidic erosion technique (A. SHARPE, 1964, personal communication).

Figure 5.9. Secondary emission electron micrograph (X 1800) of the surface of the incisal attritional facet of an adult human upper lateral incisor.
The surface was bombarded with 35keV Ar^+ ions and the image formed by the secondary electrons emitted. There are repetitive elements in the image at approximately $\frac{1}{4}$-$\frac{3}{4}$u intervals running from NE to SW, and at 1-3μ intervals running from NW to SE. It is possible that the fine surface structure (at $\frac{1}{4}$-$\frac{3}{4}\mu$ repeat intervals) is related to etching that occurred during the ion bombardment .

Note the distortion in the image about 3cms. above the centre of the bottom edge of the field which was caused by a major deficiency in the gold conducting film after it had been partly eroded away from the whole surface by the argon ion bombardment.

(TRÜB-TÄUBER "METIOSCOPE")

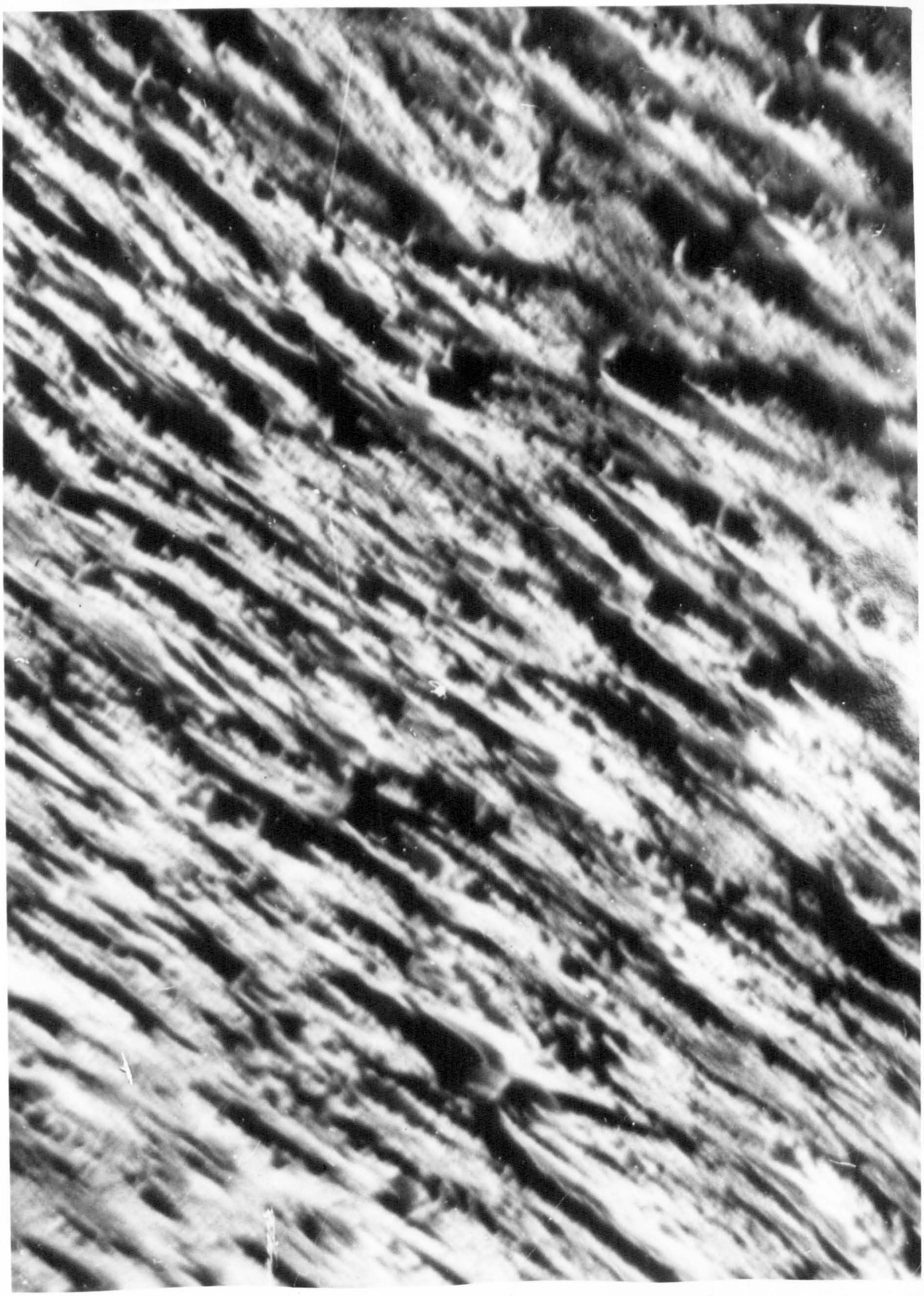

Fig. 5.9.

Figure 5.9. Secondary emission electron micrograph of attritional facet on human incisor (X 1800)

5.13 5.9. Secondary emission electron microscopy

5.9.1. INTRODUCTION, MATERIALS, and METHODS

A surface under ion-bombardment emits "secondary" electrons. These electrons can be accelerated and used to form an image of the surface: the "METIOSCOPE"* works on this principle. The specimen surface is in a position equivalent to the filament in the conventional transmission electron microscope: it is bombarded by a beam of (inert) gas ions.

The specimen must be a good conductor. A conducting film must be applied to the surface of non-conducting specimens, (e.g. teeth). The specimen surface must be as flat as possible in order to avoid distorting the electric field above the surface. Further technical details will be found in the review by WEGMANN (1961).

Specimens. In order to have a specimen with a very smooth, flat finish, a portion of an incisor was prepared so that the natural incisal attritional facet formed the specimen surface (Fig. 5.9.). This was held in a cavity in the front surface of a standard specimen holder by packing dental amalgam around it until a reasonably flat end had been achieved. (The surface of this same specimen was later polished flat; etched with dilute HCl; given a new gold conducting film; and re-examined in the METIOSCOPE -Fig. 5.10).

Another human enamel fragment was packed round with amalgam, and, after setting, the front surface (of the brass specimen holder, amalgam and enamel) was polished flat, and then lightly etched (30 secs. 0.1N HCl) (Fig. 5.11).

Conducting Films. A layer of aluminium ($\sim$ 300 Å) was applied to the surfaces after preparation. This film was sputtered away too rapidly under ion-bombardment: it was replaced by a layer of gold approximately 2000 Å thick.

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* FIRMA TRÜB-TÄUBER A.G., AMPERESTRASSE, ZÜRICH, SWITZERLAND.

Figure 5.10. Secondary emission electron micrograph (X 4800) of the acid etched surface of the same specimen as that shown in Fig. 5.9 (i.e. the incisal attritional facet after polishing and acid etching): the prisms are "sectioned" transversely in this surface. There is some repetitive detail in this surface which could represent the etched "ends of prisms". The specimen surface was covered with a layer of evaporated carbon (to form a conducting film) and bombarded with a mixture of positive Air ions, the secondary electrons emitted being used to form the image.
Note the distortion in the image near the top left hand corner caused by an interruption in the continuity of the conducting film, leading to a localised charging up of the surface under the ion bombardment, and a consequent distortion of the electric field at the surface.

Figure 5.11. Secondary emission electron micrograph (X 2400) of the acid etched surface of a fragment of human enamel: embedded (like the previous specimen) in the specimen holder with dental amalgam, polished and then etched. Gold conducting film. Argon ion bombardment.
Note the numerous circumscibed distortions in the image where the conducting film had become eroded away by the ion bombardment.

The above illustrations (Figs. 5.10 and 5.11) are prints of the original plate exposed in the "METIOSCOPE" (in the top copies of this thesis). Figure 5.9 is a photograph of a print from the original negative.

Figure 5.10. S.E.E/M-graph acid etched human enamel.

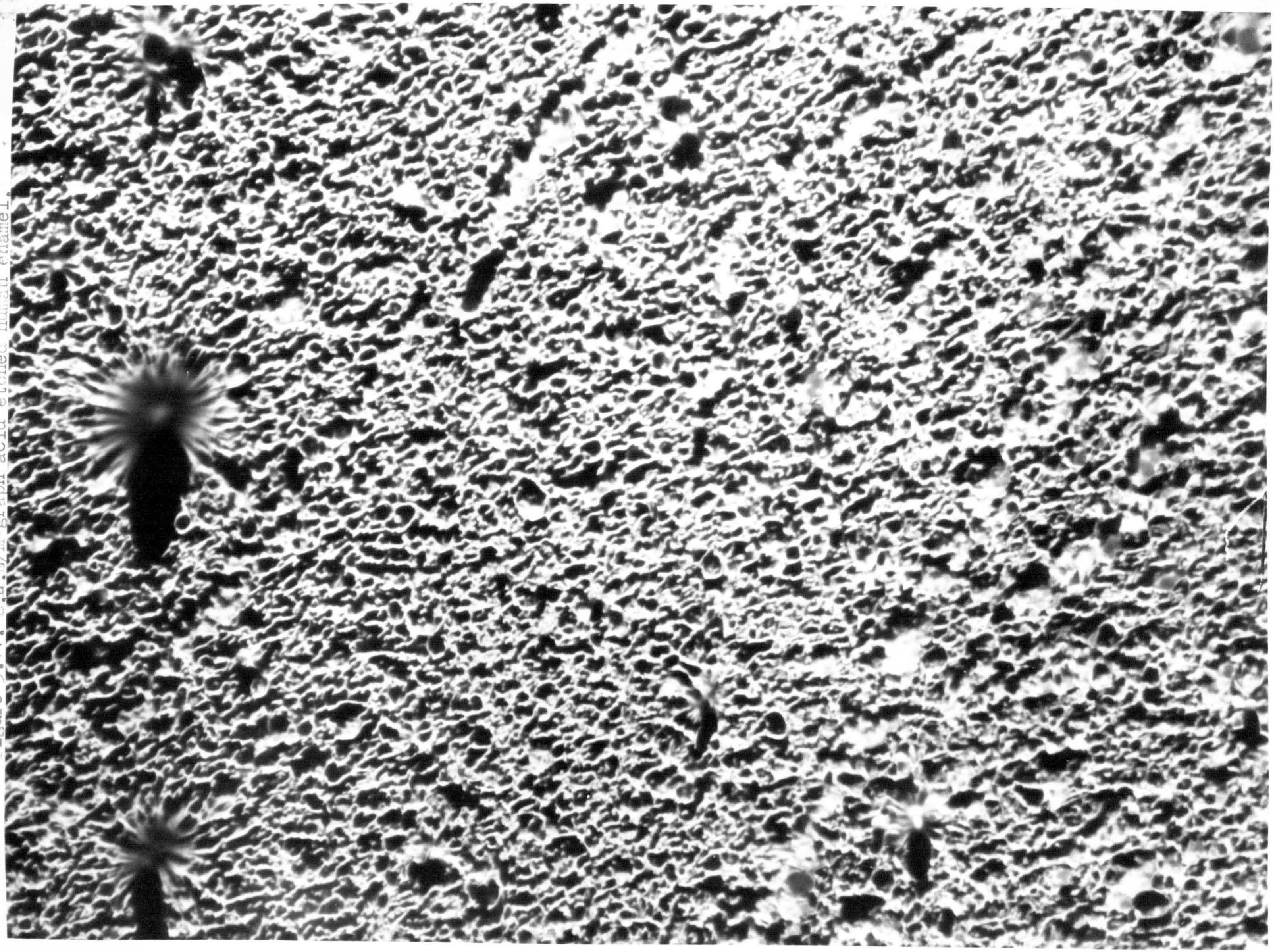

Figure 5.11. S.E.M-graph of acid etched human enamel.

5.14. 5.9.2. RESULTS The surface topography revealed in the secondary emission electron microscope (METIOSCOPE) did not resemble any of the appearances of enamel surfaces seen with other microscopical methods. The nature of the surface of the natural occlusal facet is seen in Fig. 5.9. The repetitive elements in the image, which occur at approximately 2500 Å intervals, do not appear to be related to either the prisms or the crystallites, and do not resemble the structures seen in the ion-eroded surfaces examined in a scanning electron microscope. (5.8). This surface structure, is, however, related to the sputtering erosion caused by the ion-bombardment. Fig. 5.10 shows a secondary emission electron-micrograph of the same specimen after polishing and acid etching. Repetitive features which might be associated with the prism structure can be delineated. A secondary emission electron micrograph of the other polished and acid-etched (before the application of the gold conducting film) specimen surface shows repetitive elements at $\frac{1}{2}$-1μ intervals (Fig. 5.11).

The gold conducting films were eventually disrupted by the sputtering caused by ion bombardment. The resulting "islands" of exposed (non-conducting) enamel charged by severely, causing a distortion of the electric field at the specimen surface, and hence distortions in the image. It was therefore necessary to renew the conducting film at intervals. The micrograph shown in Figure 5.9. was taken after the renewal of the conducting film - it was not possible to study the development of the sputtering damage because it occurred after the conducting film had been eroded away.

5.9.3. DISCUSSION and CONCLUSIONS. The results in this section are "very preliminary". Geographic separation from the "METIOSCOPE" has prevented an effective follow-up of the initial results.

The differences between the surface structures produced by the 20-35 kV A^+ ion bombardment of enamel in this study and the 5kV A^+ ion bombardment reported in 5.8. (BOYDE and STEWART, 1962 etc.) may be related to either the angle of incidence of the ions to the surface or to the differences in the energy of the ions employed. The angle of incidence of the ion beam to the specimen surfaces bombarded and examined in the scanning electron microscope was 45°. The ion source in the METIOSCOPE was inclined at 20° to the specimen surface, but

5.15 because of the field conditions prevailing at the surface the ions may be expected to have collided with the specimen surface at nearly normal incidence.

The absence of any detail which could be related to prism or crystallite morphology or orientation in the surface of the natural occlusal facet may well have been due to the presence of a BEILBY layer (see section 5.8.3.). Morphology reminiscent of prisms was found in limited areas of the specimens examined after the (assumed) removal of the damaged surface layer by acidic etching.

The fact that information about the true specimen surface is only obtained in the short period before the conducting film becomes discontinuous and areas of the specimen surface charge up severely makes is unlikely that this technique holds any hidden promise for enamel histology. Useful information might be obtained if the secondary electrons were emitted as a result of heating the specimen (thermal electrons) or x-ray or U-V light absorption (photo-electrons) (bypassing the problems associated with ionic bombardment)since secondary electron emission coefficients may be expected to vary with crystal orientation (WEGMANN, 1961).

5.10. MARSUPIAL "ENAMEL TUBULE" PERMEABILITY

5.10.1. In vitro dye diffision experiments. The experiments of SPRAWSON (1930) and McCREA and ROBINSON (1935-36) were repeated. The surfaces of the crowns of Macropus (species unknown-museum specimen) and Didelphis nudicaudata teeth were covered with "sticky wax" to prevent access of aqueous solutions from the external surfaces of the teeth. Their pulp chambers were then filled with solutions of basic fuchsin or crystal violet and allowed to stand until dry (1-2 days). Longitudinal ground sections of these teeth were then prepared, and examined with the light microscope.

5.10.2. Vital Dye Experiments - Trypan Blue. One male opossum (Didelphys nudicaudata) was given a series of injections of 1 and 2% aqueous trypan blue intraperitoneally. This animal was one year old and weighed 3.5 kg. He had been kept from the pouch young stage.

5.16 Methyl Blue - cavities which exposed the pulp were prepared in the buccal surfaces of some of the cheek teeth of one male opossum (one year old brother of the opossum mentioned in the previous paragraph) and one adult female opossum (age unknown; mother of previous two males). Dry methyl blue dye was 'teased' into these cavities with a probe; moistened; and the cavity sealed with quick setting Zinc Oxide and Eugenol cement (S.S. White). The teeth were extracted after an interval of one week.

5.10.3. RESULTS The in vitro dye diffusion experiments on Macropus teeth showed that both the dyes used freely entered the dentinal tubules and thence the enamel tubules; to which they remained more or less confined. Some general diffusion of the dyes through the inner layers of the enamel did occur, but the impression was gained that the enamel"tubules" could be seen as a result of, or more clearly because of, their dye content. Basic fuchsin proved to be the more satisfactory dye for "demonstrating" the enamel tubules, because it was less easily removed from them during the preparation of the ground sections.

The opossum injected with Trypan Blue finally became an overall blue colour: the blue could be seen through the skin and oral mucous membrane in particular. Ground sections of his extracted teeth showed no trace of a blue colouration in either the dentine or the enamel: the teeth did not possess a blue colour at any stage.

The methyl blue dye inserted into the pulps of the cheek teeth entered the dentinal tubules and passed some one-half to two-thirds of the way towards the enamel-dentine junction. No trace of the dye could be found in the enamel tubules. Tetracyline antibiotics were administered to some of these adult opossums (see chapter 4.3. and Appendix): no sign of the characteristic yellow flourescence under ultra-violet irradiation was ever found in the enamel.

(Discussion of the results relating to the development (2.4.3. 7.) and structure (5.8., 5.10.) of "enamel tubules" as well as the previous work on these topics will be found in 9.1.)

5.17 5.10.4. CONCLUSIONS

...Marsupial "enamel tubules" are not reached via the dentine and permeated by the dyes Trypan blue and Methyl blue in the live animal. The "Enamel tubules" are reached via the dentinal tubules and permeated by the dyes basic fuchsin and crystal violet in extracted teeth.

5.11. SUMMARY (Chapter 5)

This chapter contains the details of several experimental procedures which were employed in the examination of "adult" (fully formed) mammalian enamels. Discussion of these results follows in chapters 6, 7, 8 and 9.

The prismatic structure of enamel has been visualised in several new ways, viz:- by scanning (reflection) electron microscopy and (ion-excited) secondary electron emission microscopy of acid-etched surfaces; by argon ion-beam (erosion) etching in a scanning electron microscope; and by scanning electron-probe x-ray emission microscopy of carious human enamel (BOYDE, SWITSUR and STEWART,1962).

6.1.

C H A P T E R VI

(DISCUSSION) THE DIVISION OF ENAMEL INTO PRISMS

The division of enamel into prisms depends on a regular,repetitive pattern of orientation of its constituent elements. The orientation of these elements is determined at (and perhaps by the orientation of) the mineralising front in the developing surface of enamel. The fact that differences in the orientation of the enamel crystallites exist depends on differences of orientation of the mineralising front: the latter are the results of the peculiar mode of secretion of the enamel precursors from and about projections from the ameloblasts.

The present results (Chapter 2) confirm RÖNNHOLM (1962) in the conclusion that the secretory territories of individual ameloblasts are not necessarily equivalent to prisms. It has already been mentioned (Chapter 1) that the earlier workers were struck by the resemblance between ameloblasts and prisms and that, for them, the shape was due to the "close-packing" of these respective elements. For PURKYNĚ and FRAENKEL (1835) the prisms were square in cross section; for RETZIUS (1836,1837) they were hexagonal. NASMYTH (1841) discovered the "arcade shaped " prism cross sections.* It remained to SMREKER (1905) who rediscovered (in 1903) the "arcade-shape", to consider the implications of ameloblasts (or their secretion) of hexagonal section, being converted into prisms of arcade or horseshoe section. SMREKER (1905) considered that one side of the forming prism (the side which will be closed", i.e. have a prism-sheath" and face occlusally) hardened first and pushed into the adjacent soft sides of adjacent prisms: SMREKER did not examine forming material: he used a silver nitrate "staining" technique, which enabled him to delineate the prism outlines more clearly than his contemporaries. SMREKER's views have been widely accepted (e.g. von EBNER, 1906; ORBAN,1957; NOYES,SCHOUR and NOYES,1958). WALKHOFF(1903),MEYER (1925,1935),FREIBERG(1939)and others have held that

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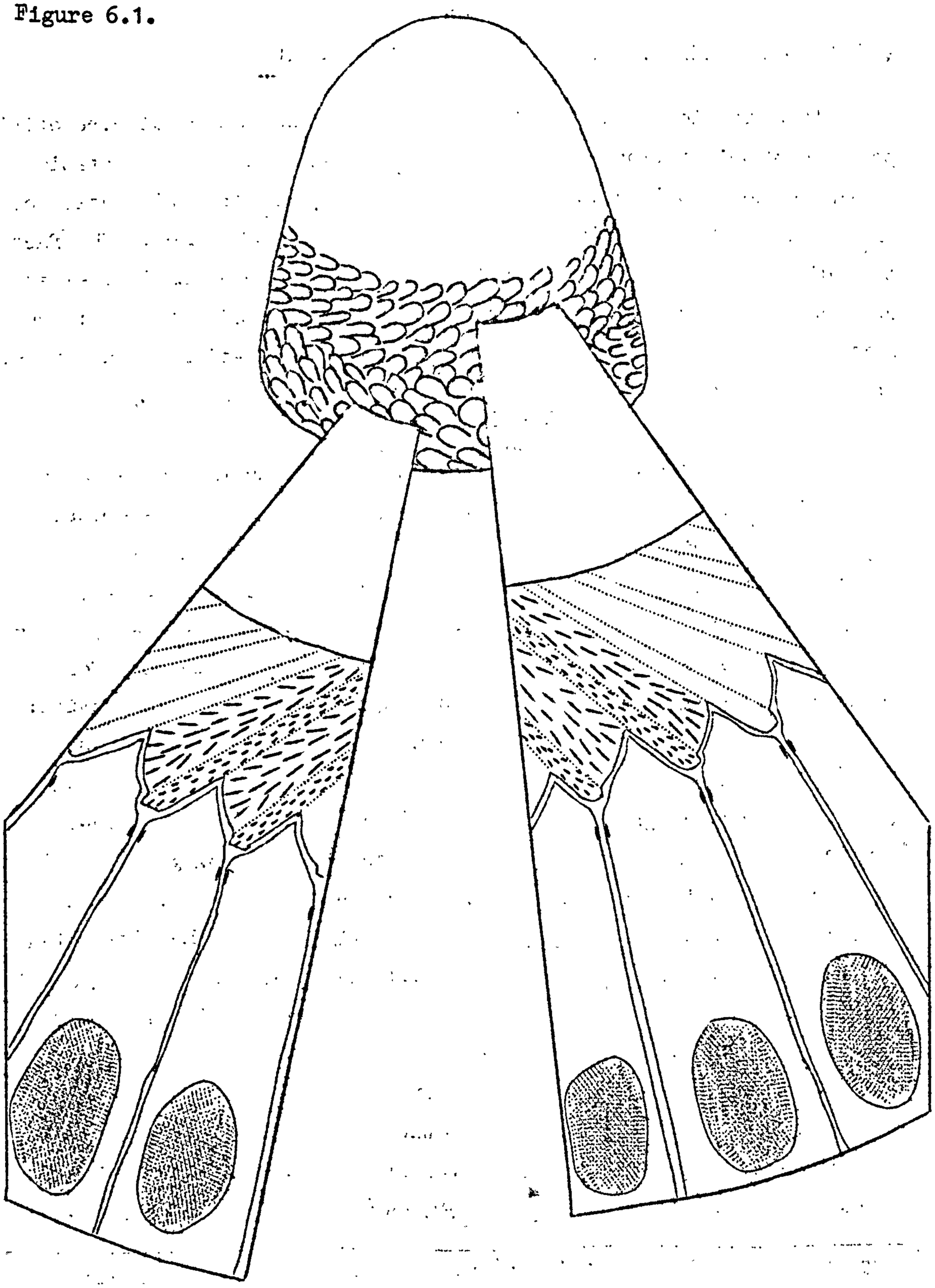
* NASMYTH actually called it"semi-circular" - NASMYTH's (1841) Figure Place C.9. is equivalent to my Figure 1.3 - PATTERN 3: and SMREKER's (1905) first arrangement ("Anordung I"). NASMYTH did not concern himself with the histology of enamel development - a regrettable omission on the part of this acute observer.

Figure 6.1.



An angle will develop between the ameloblasts and their prisms wherever zone-formation occurs. The depressions in the mineralising front associated with the development of decussating prisms fill in from alternate sides in alternate zones (almost opposite sides in carnivore enamel).

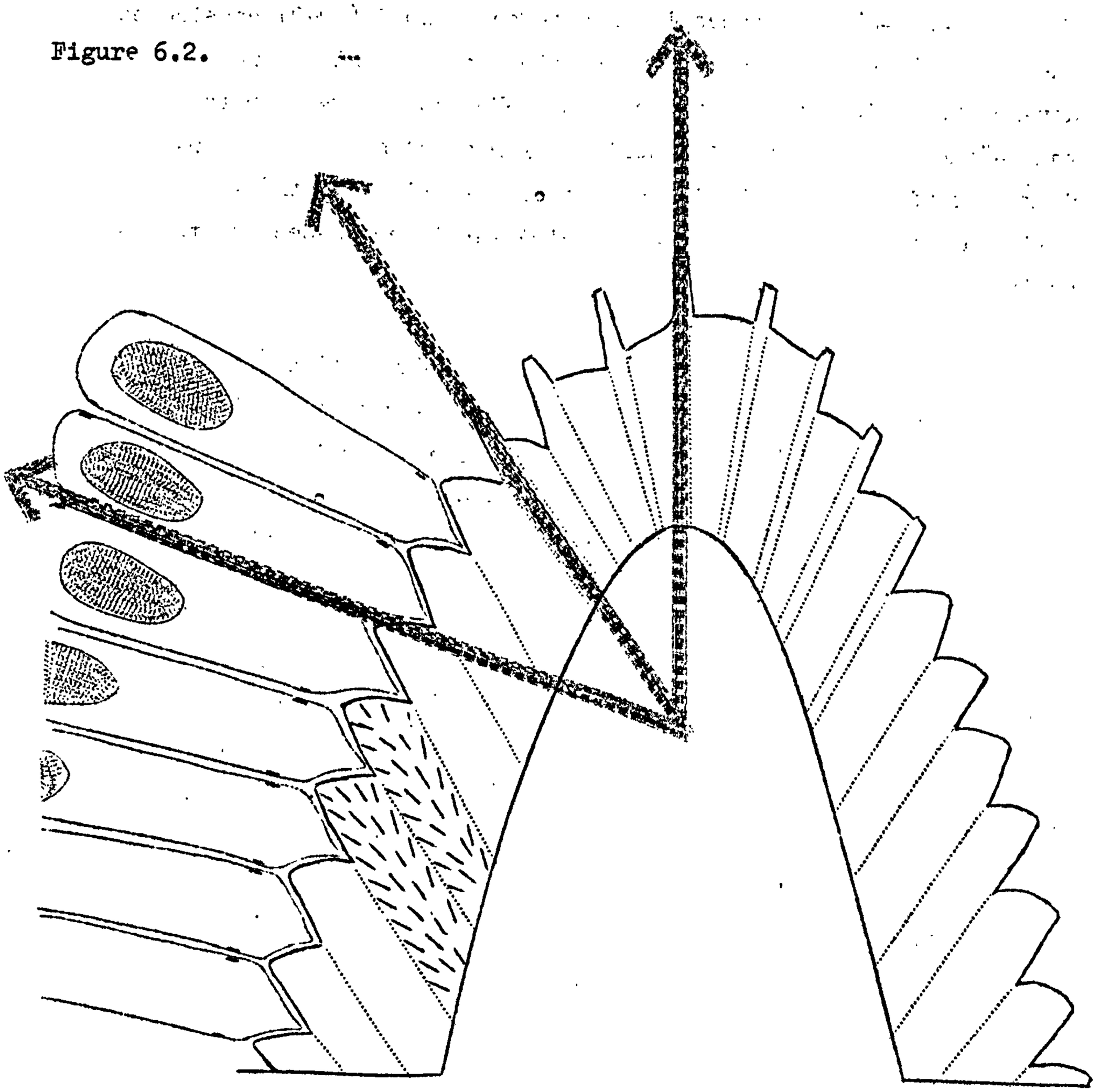
6.2.

the "open-sided" appearance is an artefact. WOLF (1940) considered the "arcade" shape of the prisms related to the angle between the developing prism and its ameloblast. GUSTAFSON (1945) wrote that -"the arcade shape is a definite formation bound to a lower degree of mineralisation of one half of the prism sheath" - and believed that the prism-sheath was circular in cross-section to begin with.

My results - which confirm those of RÖNNHOLM (1962) and FRANK and NALBANDIAN (1962) for human enamel - show that it is no longer necessary to invoke the existence of changes in shape of the prisms due to packing phenomena during development; or any relationship between the cross-sectional shape of the ameloblasts and that of the "prisms". The "prism cross-sectional shape" is the outline of the plane of sudden change in crystallite orientation (i.e. the "prism sheath" or boundary), which is determined by the way in which the depressions in the developing front "fill in". (These depressions can only be said to "fill in" with reference to a given level of time and place in enamel development - their shape, of course, remains much the same (apart from 24 hour changes - see Chap. 7.) throughout the period of active enamel deposition. They do not "fill in" and then form again anew).

The "arcade" or "horseshoe" prism cross-section (or "open sided prism sheath") is found in those prisms which fill in from one side, which occurs wherever and whenever there is an appreciable angle between the long axes of the prisms and ameloblasts. An angle may and will develop between the long axes of the prisms and ameloblasts, (1) wherever zone-formation (decussation) occurs (Fig. 6.1.); (2) where the prisms slope markedly incisally (Fig.6. 2.); (3) and/or in teeth in which the Perikymata are prominent features; i.e. where the surface of the developing enamel (the incremental line pattern) is inclined steeply against the enamel-dentine junction (Fig.6.3.). This depends on the fact that the ameloblasts tend to stand perpendicular to the developing enamel surface - the existence of an angle between prism and ameloblast depends, therefore, almost entirely on the fact that the prisms

Figure 6.2.



An angle will exist between the ameloblasts and the prism direction (i.e. the course made good by the TOMES' processes of the ameloblasts during enamel secretion) if the prisms slope incisally. That the prisms do slope incisally or cuspally is probably the result of the existence of the incisal or cuspal enamel: the secretion by the ameloblasts of the cuspal enamel will result in the ameloblasts lower cervically on the side of the cusp being pulled cuspally. Hence the cuspal slope of the prisms.

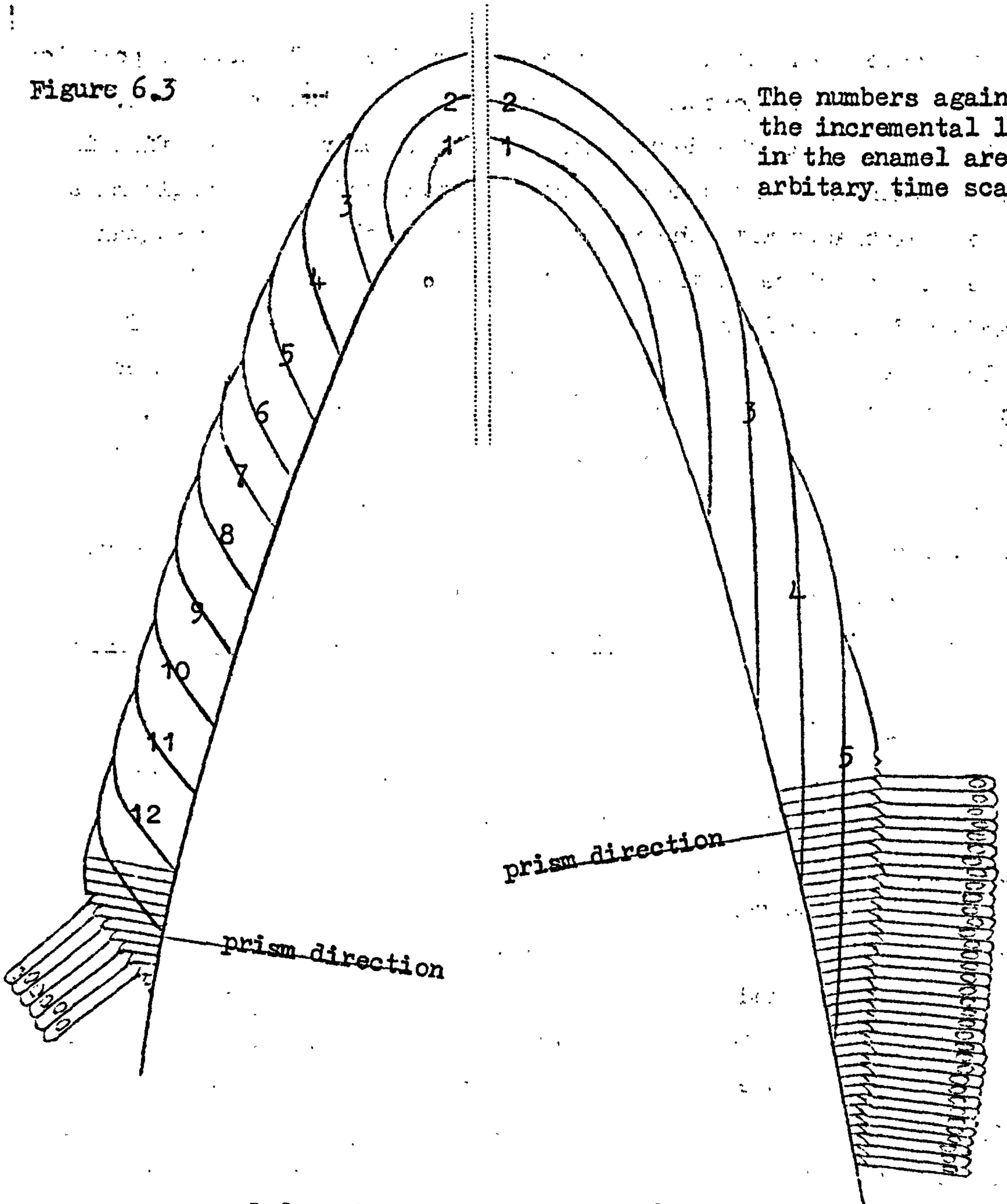
The existence of the angle between the prisms and the ameloblasts means that the prisms (strictly the depressions in the mineralising front) fill in from one side because there is less relative movement between the surface of TOMES' process and the mineralising front on this side (see Fig. 2.34.2), thence the shape of the prism boundary where the sides of the depressions in the mineralising front grow to meet each other.

6.3.

make an angle with the developing enamel surface. The actual position of the open side of horseshoe or arcade shaped prism sheaths, i.e. the side from which the depressions in the developing front fill in (more so than from the other sides) is determined by the angle made by the ameloblasts with their prisms. This angle is the resultant of the lateral slope of the prism in its zone and the vertical inwards and cervical slope against the normal to the surface of the developing enamel. There is no preference for a side from which filling in will commence if the angle is ill-defined, that is, if the ameloblast and prism axes lie nearly in the same straight line. The resultant round prisms are found over the cusp tips in human enamel, and in all the enamel of those mammals in which the prisms do not slope much against the enamel-dentine junction, nor form into decussating zones (Cheiroptera, Insectivora, Cetacea and Sirenia.)

The ameloblasts must make an angle with their prisms if they stand perpendicularly on the enamel surface, yet the prisms cross each other in alternate lamellae (zones, decussation). The angle contained between the prism and ameloblasts will be on alternate sides in alternate zones. The prisms fill in from the contained-angle side, and hence fill in from alternate sides in alternate zones. If there are well marked zones, yet not much vertical slope of the prisms - and this is the case in the lateral enamel of the Carnivora - then the open sides of the prisms face to left and right in alternate zones (SUSS,1940). In the Murinae (Rattus norvegicus,Figs.2.17., and 2. 15.1.)there is both a marked decussation and a marked vertical inclination of the prisms. The"horseshoe" outline is inclined at approximately 45^{o} to the long axis of the tooth; and faces alternately apically (cervically) and left, and apically and right in alternate zones. The same is true in human enamel, but in this case the vertical inclination of the prisms predominates and the open side of the horseshoe only inclines at an angle of something like 30^{o} to the transverse plane of the tooth.

Figure 6.3



The numbers against the incremental lines in the enamel are an arbitary time scale.

Back 6.3

LEFT: when enamel deposition is a long and drawn out process the surface of the developing enamel is inclined strongly against the future enamel-dentine junction. (Assuming that the prisms make the same angle with the enamel-dentine junction) the ameloblasts will be more inclined against the direction of the prisms in this case, than RIGHT where the enamel coverage of the crown surface is rapidly completed and the surface of the developing enamel is almost parallel to the enamel-dentine junction.

6.4.

The fact that prisms in general do not stand normal to the enamel-dentine junction, but tend to slope towards the incisal edge or cuspal tip, is probably due to the very existence of the incisal or cuspal enamel. That part of the ameloblast layer which secretes the cuspal enamel "pulls" the rest of the ameloblast layer (lower down on the sides of the tooth) with it, and so determines that the path described by the TOMES' processes of the ameloblasts is inclined incisally (cuspally) against the enamel-dentine junction. The resulting (contained) angle between the ameloblasts and the prisms must face cervically - the prisms(i.e. the depressions) are filled in from the contained-angle side; that is from the cervical side. The open sides of the prisms face cervically in the formed enamel.

The prisms are also inclined against the ameloblasts in the sense that they slope to left and right in zones (except in the Cheiroptera, Insectivora, Sirenia and Cetacea). The angle between the ameloblasts and prisms may be greatly increased if the slope of the developing enamel surface is strongly inclined against the enamel-dentine junction (as is the case where enamel development is a long and drawn out process as, for example, in man (Fig. 6. 3.).

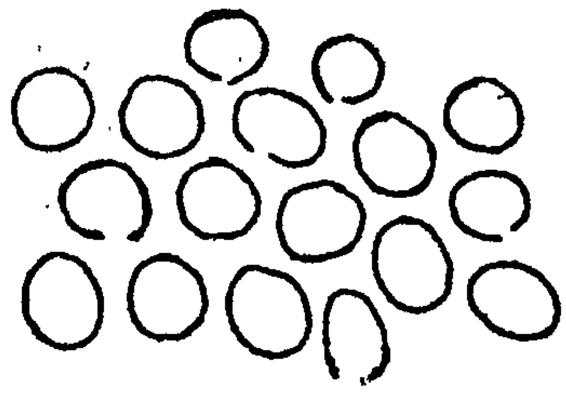
The most comprehensive survey of the shape of the enamel prisms in the different mammalian orders is that of SHOBUSAWA (1952). This author distinguished five major prism cross-sectional patterns viz:- Primate, Carnivore, Ungulate, Rodent and Cetacean. SHOBUSAWA's Primate (and Proboscidean) type corresponds to the "semicircular" prism(sheaths) of NASMYTH (1841) i.e. PATTERN 3 (Fig. 1.3. - the "arcade" shape with "winged processes" of von EBNER (1906), MUMMERY (1916,1919), CHASE (1927 A, 1929) and FUJITA (1953). FUJITA also considered PATTERN 3 to be the most common in human enamel). Primate enamel is also characterised by narrow interprismatic regions.

SHOBUSAWA's Carnivore prism-type is hexagonal in cross-section - exhibiting hexagonal close-packing, more interprismatic substance and much more extensive prism-sheaths. Complete prism sheaths are common in the dog (MEYER,1925; FUJITA,1953) and less common in cat enamel. The latter has more "interprismatic substance" than the former.

Figure 6.4. The position of the open side of the prism boundary is within the angle contained between the prisms and their ameloblasts. This angle is the resultant of the lateral slope of the prism in its zone and the vertical inwards and cervical slope against the normal to the surface of the developing enamel.

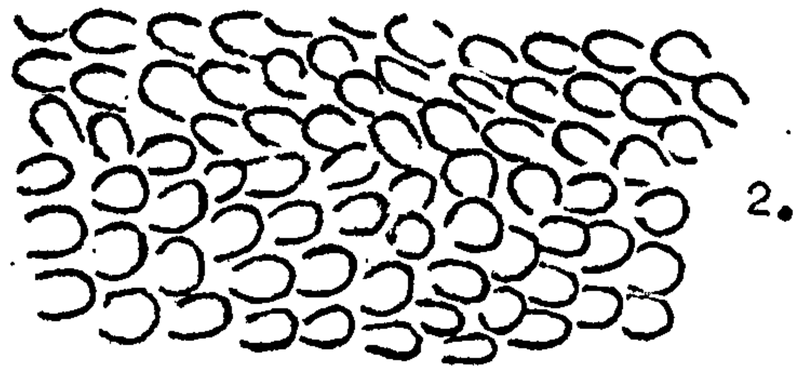
1) a. no decussation.
   b. limited cuspal slope of prisms.
   c. enamel formation not unusually drawn out, i,e. incremental lines almost parallel to E-D. J.

e.g. manatee.


1.

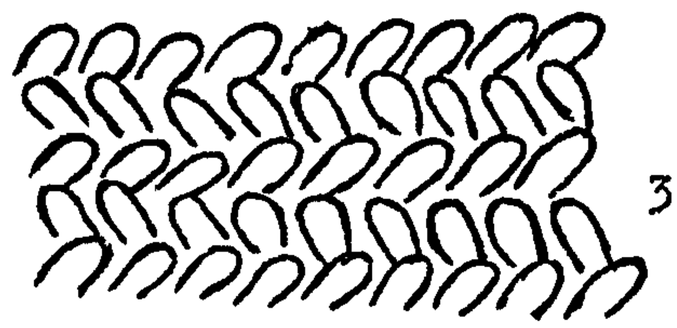
2) a. marked decussation (ca. 60°).
   b. no cuspal slope of prisms.
   c. enamel coverage very rapid, i.e. incremental lines almost parallel to enamel- dentine junction.

e.g. dog.


2.

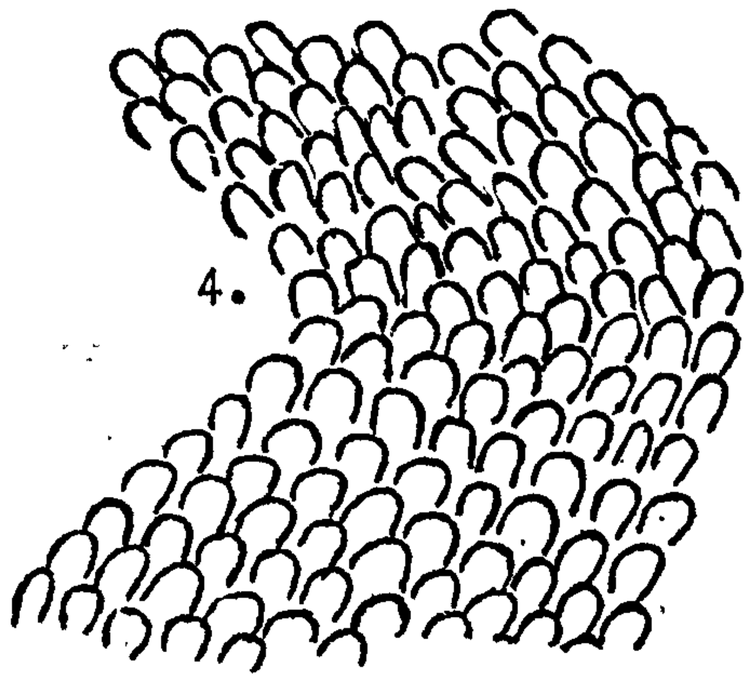
3) a. marked decussation.(ca. 90°: single transverse rows = zones).
   b. marked incisal slope - 45°.
   c. enamel coverage very rapid.

e.g. rat incisor inner-enamel.


3.

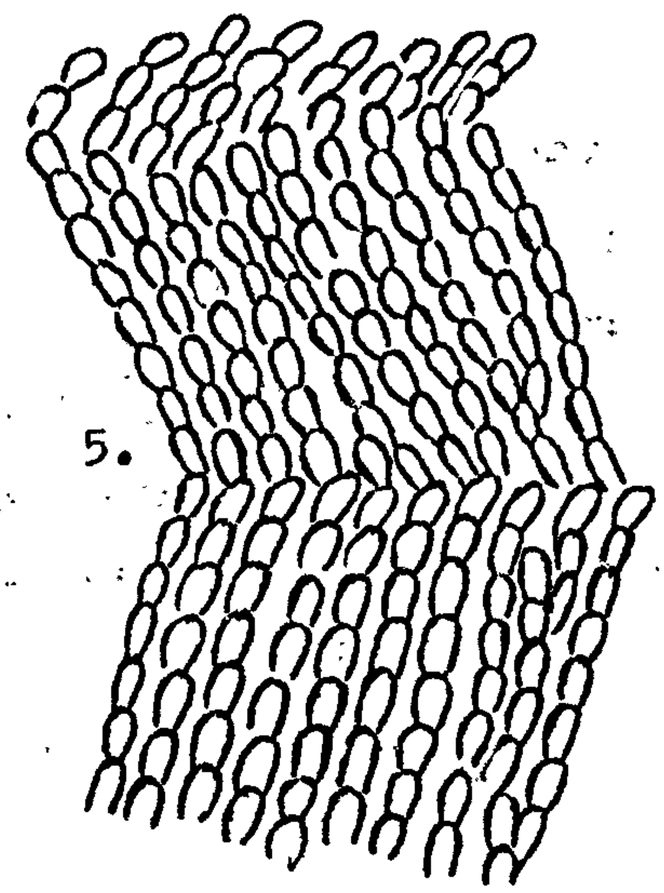
4) a. moderate decussation: prisms cross at ca. 45°,
   b. moderate cuspal slope (ca. 70°).
   c. enamel coverage very slow, i.e. incremental lines steeply inclined against E-D.J.

e.g. human permanent teeth.


4.

5) a. decussation: prisms cross at ca. 45°.
   b. marked cuspal slope of prisms against E-D.J. - ca. 45°.
   c. enamel coverage very rapid, i.e. incremental lines almost parallel to enamel-dentine junction.

e,g. cow.


5.

6.5. The Ungulate prisms are ovoid in cross-section and are organised into well ordered longitudinal rows - the length of the ovoid lying in the row. There is a great deal more interprismatic substance between prisms in adjacent rows than between prisms in the same row.*

The Rodent type of prism cross-section doubtless depends on the mode of decussation of the adjacent rows (lamellae) of prisms. The best descriptions are those of KORVENKONTIO (1934 - 35). He found that the cross-section of the rods in the incisor inner-enamel of the Scuiromorpha was almost square : of the Myomorpha was flattened rhomboidal and of the Hystricomorpha very variable - in fact described as comma, spindle, triangular or quite V-shaped by SHOBUSAWA. The shape of the rods in various rodents has been described by ERDL(1841),TOMES (1850) and von EBNER (1906), amongst others.

The last characteristic type of prism cross-section described by SHOBUSAWA is found in the Cetacea (Odontoceti - dolphins), Insectivora, Cheiroptera (LOHER ,1929) and Sirenia (dugong, - SHOBUSAWA: manatee - BOYDE). The prisms are often round, with complete sheaths, and there is abundant interprismatic substance. SHOBUSAWA stated that the prisms of some bats closely resemble those of the Cetacea and others occupy a position midway between those of the Cetaceans and Ungulates.

SHOBUSAWA's work is undoubtedly of great value, but it cannot be entirely agreed that "the form of the enamel rods is species specific" and that - "the patterns of the rods in cross-sections are constant for each order of mammals". The edges between the patterns encountered in the different mammalian orders are definitely blurred. SHOBUSAWA himself commented that the rods in the Carnivora - "may also present (the) scale-like appearance" seen in the Primates and Proboscidea. He also figured almost round prisms with abundant interprismatic substance in Felis domesticus and Elephas indicus, and it is a matter of common knowledge that this pattern frequently occurs in human teeth over cusp

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*This organisation has also been mentioned or figured by:..... WILLIAMS (1896 A p.478, Fig. 83 and caption); WEIDENREICH (1926 p.326 Fig.23, in the horse); by SHOBUSAWA (1952),MUMMERY (1916), CARTER (1922), and WILLIAMS (1923 A) in Macropus ;by MARCUS (1931 Fig.18 p.412) and HAUSELE (1932 Figs. 2 and 3) in Didelphys paraguensis; and by LUDWIG (1941) in the rabbit (Fam. Leporidae,Ord.Lagomorpha).

6.6.

tips: nevertheless, he performed an indispensable service in making these comparative observations and with these reservations I am prepared to substantiate his "classification" from my own observations. The common occurrence of a particular feature of the shape of the prisms probably indicates common properties of the relationship of the ameloblasts to the prisms and to their surrounding environment.

The existence of "prisms" in enamel depends on the pattern of orientation of its constituent elements, particularly the crystallites; and very particularly on the presence of planes of abrupt change in crystallite orientation - the "prism-sheaths": this is widely accepted. However, there is no universally accepted interpretation of the light microscope image of ground sections of enamel in terms of the commonly accepted hypotheses regarding its structure.

The advances which were obtained from the further application (after PURKYNE, 1835 ;RETZIUS and the LINDERERS, 1837) of the ordinary light microscope to the study of the structure of enamel were made by the application of some elementary, theoretical interpretation to the observed image. Except in the case of the iron pigment (von BIBRA,1844; DAM and GRANADOS, 1945) in rodent incisor and shrew molar enamel,(BOYDE and SWITSUR, unpublished), mammalian enamels contain no colouring matter (the colour of the "brown" striae of RETZIUS depends on the structure in these regions - see 8.1.), yet a wealth of detail can be seen in a light microscope image. This detail must be determined by the presence of sharp gradients of change of refractive index within the specimen. Refractive index varies (as an approximation) with the density of the medium and this elementary knowledge was applied by many early interpreters (e.g. von EBNER, 1890; WALKHOFF, 1895).

Since it is known that enamel consists(reduced to the simplest terms) of a mixture of <u>water</u> (refractive index, n = 1·33) <u>organic material</u> (from data provided by BARER (1956), a 100% solution of protoplasm, i.e. a mixture of carbohydrate, fat and protein, would have a value of n ≃ 1·51) <u>and hydroxyapatite</u> (n = 1·62), it has been natural to assume that the variations in refractive index which underly the possibility to achieve contrast in the light microscope image are

6.7.

related to variations in the ratio of mineral to organic material. Expressed in another way, it has been almost universally assumed that there are large variations in organic content within the enamel which would account for the change in refractive index at the periphery of each enamel prism which enables the "prism structure" of the enamel to be visualised.

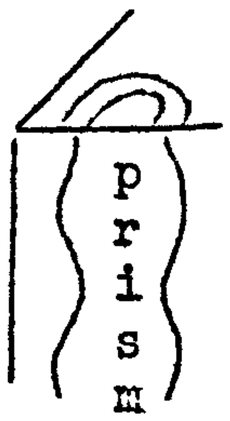
WALKHOFF (1895,1898,1903,1923) and von EBNER (1890,1903,1906) argued in almost melodramatic terms about the existence of a separate "Kittsubstanz". WALKHOFF held that a less-mineralised(and therefore of lower refractive index) peripheral cortex surrounded each prism - von EBNER held that the lower refractive index material did not belong to the prisms but constituted a separate interprismatic cementing substance (Kittsubstanz).

Later still - after the influence of BÖDECKER's rediscovery, and oft repeated publication (e.g. 1909) of the presence of organic prism sheaths and transverse prism-septae (cross-striations) in carefully decalcified enamel - the ground-section image came to be interpreted as revealing the existence of prisms of a high refractive index, surrounded by a thin, highly organic sheath of a low refractive index, and separated from each other by a variable amount of an interprismatic substance possessing a refractive index approaching that of the prisms proper. Likewise, the appearance of the cross striations of the prisms in the light microscope image has been held to indicate changes in the organic content (refractive index) of the prisms themselves(ORBAN,1951).

The microscope in which image contrast is determined by (small) changes in refractive index (over limited distance) within the specimen, is the phase-contrast light microscope. Although phase-contrast microscopy was available as a practical technique in the late 1940's, the considered, careful application of this method was not reported until FREMLIN and MATHIESON (1962) published their observations. These authors showed that the "horseshoe" appearance of the cross-sectional shape of most human enamel prisms is real (This had been confirmed by many careful workers since LINDERER and LINDERER (1837): see JASSWOIN(1925), FUJITA (1953) and most electron microscope workers). FREMLIN and MATHIESON showed that on the side of the prism possessing a prism boundary (i.e. the horseshoe outline itself) there is a large,sudden

6.8. change of refractive index between the prism and the interprismatic substance, while on the other side there is not: they wrote that - "This requires that there should be a gradual change either along the diameter of an individual prism or through the interprismatic substance. On the basis of visual observations we are inclined to believe that the change is within the prisms rather than outside. More refined methods of observation should settle this when the equipment is available". FREMLIN and MATHIESON's contribution must be regarded as a major advance in the field of interpretation of the light microscope image in enamel studies. It is interesting to note that they do not make much of a "prism-sheath": they accept what they observe, viz., that there is a large,sudden change of refractive index on one side of the prism.

The refractive index of enamel may vary with the orientation of the (birefringent) crystallites as well as with changes in the proportion of organic material. Most previous workers have assumed that the latter factor is entirely responsible for the observed changes in refractive index. It cannot be denied that more organic material can be recovered from "prism sheath" regions in decalcified material, but the light microscope does not give valid information about the width of this plane (i.e. the prism boundary). Electron-micrographs of decalcified sections of adult enamel show that the "prism-sheath region" is not more than 0·2 μ wide, yet the concept has been allowed to creep in that the prisms are surrounded by veritable masses of organic material - sufficient for HODSON (1952) for example, to state that he preferred the term "rod sheath", because of its implications of a greater thickness for this region. There can be little doubt that the information contained in the light microscope image of a ground section of enamel is derived from a considerable thickness of material, that is to say that summation effects must play an important part in determining image contrast and topography. A variation in the width of the prism from the artificial concept of it as a straight, smooth cylinder - such as must be envisaged if we call the cross-striation varicosities - will be appreciated as an apparent increase in the width of the "prism-sheath" when the prisms are looked at end-on, i.e. in transverse section.



6.9. It is possible that a major component of the real difference in refractive index (observed by FREMLIN and MATHIESON, 1962) between the regions on either side of the ("horseshoe shaped") prism boundary planes is only the difference between the refractive indices of the crystallites when viewed along different directions: CARLSTRÖM and GLAS (1963) give the birefringence of apatite crystallites as (-) 0.007: FREMLIN and MATHIESON (1964) have very recently found differences of "up to 0.02" between the refractive indices of the "prism cores and interprismatic regions". Only a very small change in composition between these regions would be needed to account for the difference in their refractive indices. There is, however, a real difference in composition <u>at</u> the boundary plane and unless this "plane" is imbibed with a medium of refractive index approximating to that of the apatite crystallites themselves, it forms a highly reflecting surface within the enamel. This manifests itself as a bright or dark line depending on the conditions of viewing, i.e. whether light is added to, or subtracted from, that forming the image. It is not necessary to suppose any great width for such a reflecting plane (it can be a small fraction of the wavelength of the radiation used to form the image - some actual values calculated by Dr. M.BRADEN for the fraction of light reflected at such narrow planes are given in Appendix C): its apparent width depends on its passing obliquely through the ground section, and on its own, corrugated course.

Since the existence of prisms in enamel depends on the pattern of orientation of its crystalline elements, it would be as well to consider the limitations of the methods which have been used to study this pattern.

The birefringence of enamel implies the existence of an ordered structure. Polarised light microscopy revealed the existence of an ordered structure - the ordered elements being below the limits of resolution of the light microscope - and showed that these submicroscopic elements were neither parallel to the prism, nor to one another across the diameter of a prism (von EBNER, 1890).

The determination of the orientation of the apatite crystals is a three-dimensional problem to which the polarised light microscope only contributes two dimensional information (extinction directions).

6.10. It follows from this that it must be impossible to determine (accurately) the orientation of the crystals in enamel, even supposing that they were parallel to each other throughout the thickness of a section: from this we may deduce that variations in the observed birefringence due to changes in crystal orientation cannot be entirely accounted for, and that it is therefore impossible to derive other than questionable deductions about variations in the concentration of apatite from polarisation microscopy and measurements of birefringence alone.

WYART and TOURNAY (1949),HELMCKE (1959,A,B),CARLSTRÖM(1960), TORELL (1960,A,B.), CARLSTRÖM and GLAS (1963), ANGMAR,CARLSTRÖM and GLAS (1963) and CÀRLSTROM,GLAS and ANGMAR (1963) have all sounded well -founded warnings about the limits of applicability of polarisation microscopical methods to the study of enamel: varying from the fact that extinction directions can only be determined approximately,(WYART and TOURNAY, 1949); to pointing to variations in the effect of imbibing fluids of the same refractive index, depending on their chemistry (SCHMIDT, 1923 - 25; TORELL, 1960, A and B). Polarised light microscopy did, however, provide the first information about the orientation of the crystallites within enamel prisms, albeit approximate information. The method has the advantage of simplicity in operation, if not in interpretation- and it allows <u>average</u> results from enormous numbers of prisms to be obtained: i.e. the general pattern of organisation of enamel can be studied. It is therefore an indispensable adjunct to the more recent "high resolution" methods with their more limited fields of view.

The method of extinction direction determination(between crossed nicols) suffers from the disadvantage that the particular crystallite orientation that is determined is an "average" for the whole thickness of the section contributing to the relevant image point. The proportionate distribution of all the crystallite orientations present can be determined using x-ray diffraction methods : the main disadvantages of which are that no"image" is formed, and that it is not possible to relate the information about crystallite orientation to actual structures within the specimen. However, it is possible to reduce the size of the specimen examined,and hence to relate the information obtained to smaller regions of the original sample (CARLSTROM,1960 -"micro-x-ray diffraction"-Chap.10).

6.11. Electron-microscopy of ultra-thin sections - combined with the use of stereo-techniques and electron diffraction - is a very powerful method for studying crystallite orientation in enamel. The principal objections to be raised against the validity of the results are on the grounds of the distortion of the original organisation of the tissue which occurs during the preparation of sections. This is the only method which can relate crystallite orientation to morphological position in the enamel with high lateral resolution (inherent in the electron-microscope) and high resolution in depth (because the sections are very thin).

Indirect information about differences in crystallite orientation can potentially be gained from physical and chemical etching studies - "destructive testing"! in the modern nomenclature. Differently disposed crystallites are eroded away at different rates during argon ion-beam bombardment (BOYDE and STEWART, 1962), "airbrasive" bombardment (FEARNHEAD and BOYDE, unpublished) and acid etching (viz:- the increased visibility of the"prisms", their "cross striations" and the HUNTER-SCHREGER bands).

Microradiographic and x-ray emission microanalytical studies have not revealed any (quantitative) differences in the mineral content in prism and interprismatic regions : "prism structure" must therefore be regarded as dependent mainly on the pattern of orientation of the crystallites (particularly on the existence of planes of abrupt change in orientation), until evidence to the contrary becomes available.

von EBNER (1906) and MUMMERY (1916,1919) both described the separation of a "membrane" (human - von EBNER :" membranous expansions", of MUMMERY in Macropus) of interprismatic substance in teased preparations of developing enamel. This is good evidence of the degree of functional separation of adjacent longitudinal rows of prisms. MUMMERY's "membranous expansions" are almost certainly equivalent to the interrow (interprismatic) sheet regions in PATTERN 2 enamel (Fig.1.2.). Previous workers have taken very little notice of the formation of rows of prisms. The acceptance of the formation(and function as such) of rows of ameloblasts on the scale necessary to account for the formation of the

6.12. HUNTER-SCHREGER bands - which may consist of annular layers of say 10 prisms in man and dog - is the first step towards visualising the single prism-rows "lamellae"of TOMES, 1850: Pseudoprismen of KORVENKONTIO,1934) and hence the single ameloblast-rows in rodent incisor inner-enamel formation. In this latter situation one must imagine that alternate rows of ameloblasts slide past each other in opposing directions in order to begin to account for the characteristic arrangement of (decussating)prisms. Having accepted this as possible, it is no great step to accept that the organisation of ameloblasts into rows is a general tendency of these cells and need not only be manifested when the rows of cells move past each other for some unknown reason.

Function of Structural Organisation of Enamel. The physiological significance of the structural organisation of enamel might be considered in the following terms. Enamel presumably subserves the function of providing a hard, abrasion resistant, tough, protective covering to the basis-substance of the teeth. Enamel must be as hard - or better, harder than - all the animal and vegetable tissues which are comminuted as a preliminary to the digestive process. Apatite is the hardest and the least soluble mineral found in biological systems (other than the mineral fragments which are ingested and utilised whole in some organisms); and it is therefore no surprise that enamel contains a great deal of apatite. It is impossible to envisage a biological system which could form an enormous single crystal of an apatite, but it should not be forgotten that considerable advantages are conveyed to enamel by virtue of the fact that it is not a single crystal. It is recognised that the elusive property of "toughness" (resistance to brittle fracture) in a crystalline material is largely determined by its crystal ("grain") size, and that within limits, the "toughness" is increased with decreasing crystal size (i.e. increasing polycrystallinity). Put in rather elementary terms, this is because : 1) smaller crystals are more "flexible" individually (consider, in this connection, the curvature which may be suffered by the very fine diameter crystals at the mineralising front of the enamel in the ultra-thin sections of this region; as compared with the greater proportion of fractured, larger diameter crystallites seen in the deeper

6.13

portions of the developing tissue): and 2) because any potential fracture (cleavage) of the crystallites is contained within narrower boundaries. A fracture at any point would have to extend throughout the whole tissue, were enamel to consist of one large crystal: in other words, it would be exceedingly brittle. Hydroxyapatite possesses very poorly defined cleavage planes although it belongs to the hexagonal system in which basal-plane cleavage is generally predominant. Nevertheless, a structure made up of a parallel array of elongate crystals would be expected to cleave in directions parallel to the long axes of the crystallites in this array. This difficulty has been overcome in enamel by the factor which might be called poly-orientation : the presence of many different orientations of the hydroxyapatite crystallites in enamel will presumably limit the progress of any potential cleavages between adjacent crystallites. The poly-orientation may be seen on one scale in the change in orientation of the crystals across individual "prisms" and within the "interprismatic regions" and, on a different scale, in the mutual interweaving or decussation of the bundles of crystallites (i.e. prisms) in the gnarled enamel over cusp tips and in the HUNTER-SCHREGER bands, respectively.

HELMCKE (1958,B) suggested that the feather-like arrangement of the crystallites within the prisms may confer a degree of deformability and elasticity upon the tissue, which it would not otherwise possess. His model arrangement (loc. cit. Fig. 10. p.54) is certainly quite convincing in this respect, but it implies that the organic matrix must permit a little movement of adjacent crystallites in respect to each other. This is not unlikely, since one would imagine that the organic components of the matrix are more deformable than the apatite: HELMCKE does not seem to have commented on this matter himself. PERDOK (1963) has suggested that a part of the property of resistance to brittle fracture of the enamel resides in the organic component of the matrix; which would in some way contain a part of the energy transmitted to the enamel during a mechanical shock, and disperse it more gradually and significantly later.

6.14. SUMMARY (CHAPTER VI.)

The existence of "prisms" depends on the pattern of orientation of the crystallites - itself dependent on the formation of TOMES' processes during development - and their appearance in both the light microscopic and electron-microscopic image depends on the planes of interruption in this pattern, i.e. the prism sheaths. The large apatite component in enamel confers upon it the necessary hardness; and the concomitant risk of brittle fracture is avoided by its poly-oriented poly-crystallinity and the presence of the organic matrix.

C H A P T E R VII

PRISM DECUSSATION or ZONE FORMATION.
(THE HUNTER-SCHREGER BANDS - DISCUSSION)

PURKYNĚ and FRAENKEL (1835) and LINDERER and LINDERER (1837) realised that the enamel prisms are formed into extensive groups which form horizontal girdles about the tooth, such that in each girdle (corresponding very approximately to a transverse section through the enamel of the middle of the crown of a tooth) all the prisms have the same direction; which is opposed to some extent to the course of the prisms in the next successive girdle, but matched in the next-but-one girdle (belt, girth, Gürtel, layer, lamella, disc, band, stripe, zone, etc.) and so on. It was appreciated, then, from the first discovery of the enamel prisms (fibres) that they may cross over one another in the form of an X, which act is appropriately described by the verb to decussate. The term was first applied by OWEN (1845, p.406) who wrote of beaver incisor enamel -"the fibres of the inner half being cut across give the appearance of fine decussating oblique lines;" ...(ERDL (1841) correctly described the disposition of the"fibres" in rodent incisor enamel; TOMES (1850) provided the first exhaustive account). A longitudinal section through a tooth exposes successive layers of prisms cut at different angles - the prisms in alternate layers being cut at similar angles. Light is scattered differently from the differently disposed prisms. The layers of prisms can be seen with the naked-eye because they are thick enough, and because they scatter light differently according to the arrangement of their prisms. The layers of prisms in human enamel are called HUNTER -SCHREGER bands, after HUNTER (1761 - Latin treatise - cit. BLANDIN, 1836; 1770 1st. ed. and 1778 2nd ed. in English) and SCHREGER (1800). However, equally apposite descriptions had been given by GAGLIARDUS (1689 = date given by HENLE, 1841 and MAGITOT, 1858: the edition I consulted was dated 1723), DUVERNEY (cited by NASMYTH, 1839 after RETZIUS, 1836 - the latter gives the date as 1639, but COHEN, 1963, personal communication, assures me that this date is wrong, as the relevant journal was not in existence at this

7.2.

date), LEEUWENHOEK (1678, etc., cit. SCHIERBEEK, 1959), MALPIGHIUS ("Anatomia Plantarum". Op. omn. Lugd. Batav. (1687) cit. NASMYTH, 1839A: Opera Posthuma, 1697), HAVERS (1689), de la HIRE (1699), WINSLOW (1733), FAUCHARD (1746, description is after de la HIRE); LUDWIG (cit. BLANDIN, 1836) and BERDMORE (1770 - "New" edition. First edition was in 1768). The "fibrous texture" was also recognised by HERRISANT (1758) ; de la FIÉRE (ca. 1750 - 1760 cit. BLANDIN,1836) and BROUSSONET (1787).

Several different explanations of the various optical appearances of the HUNTER-SCHREGER bands have been forwarded; but all the descriptions are more or less in agreement that they can only be seen in longitudinal sections of teeth, and are much more easily seen when viewed by reflected light. The white (more reflecting)bands seen in longitudinal sections of enamel at low magnifications correspond with zones in which the prisms lie with their long axes approximately in the plane of the section and the darker bands correspond with zones of more transversely sectioned prisms.

LODGE (1917) considered that "lines of SCHREGER (were) due ..... to net effects produced by the light falling upon the cut enamel prisms and the optical densities varying according as the mesh of these nets are in apposition or in interference," or in other words, that the HUNTER -SCHREGER bands are MOIRÉ patterns of a sort. We are left unaware, however, of what we are to regard as the superposed "nets" in this ingenious explanation. LODGE considered that "the lines of SCHREGER are wider at the periphery than at the amelo-dentinal (junction) ends by a ratio comparable to the diameter of the rods at these ends respectively".

Before attempting to give an explanation of the appearance of the zone-pattern of the prisms in the light microscope image, it would be as well to mention two quite pertinent observations- the relevance of which will be self-apparent in a moment. First , CZERMAK (1850) noted that the brown striae of RETZIUS (see section 8.1.) did not continue uninterruptedly over the whole crown but that..."the colour suddenly or by degrees stops at certain places". Close examination

7.3. of longitudinal ground sections will reveal that the intensity of the colour of the brown (in transmitted light: blue in "reflected" light) striae varies in alternate (HUNTER-SCHREGER band) zones (I consider it most probable that this is the phenomenon to which CZERMAK referred). Second, von EBNER (1890) noted that the prominence of the HUNTER--SCHREGER bands was reduced on boiling a ground section (he referred in particular to the dark("brown") bands seen in transmitted light): this he considered due to the removal of air (or gas) from "spaces". Alternate HUNTER-SCHREGER bands(more precisely the parazones(PREISWERK, 1895) containing more or less longitudinally sectioned prisms) are indeed "brown" when examined by transmitted light; these same darker (in transmitted light)zones appear blue-white by reflected light* - the same phenomenon is manifested by the brown striae of RETZIUS(and is considered in more detail in that context - see section 8.1 and Appendix C), and the explanation of the formation of the "colour" is undoubtedly the same in both these cases, viz: that shorter (blue) wavelength visible radiation is scattered in preference to the longer wavelengths. Hence blue light is scattered (and removed) from the transmitted beam, which, therefore, appears more yellow or orange. The significance of these considerations in relation to the HUNTER-SCHREGER bands is that their appearance implies that the scattering effect is strongly dependent on the orientation of the light- scattering "particles", i.e. the crystallites or the intercrystalline spaces.Light is only scattered where it passes through "parazones" in which the crystallites are oriented perpendicular to the direction of propagation of the beam: it is unaffected in the diazones of transversely sectioned prisms , where the crystallites are parallel to the direction of propagation. The predominantly parallel array of the crystallites and intercrystalline spaces in enamel would lead one to suspect that the scattering effect is dependent on the plane of polarisation of the incident radiation. The visibility of the HUNTER-SCHREGER bands is

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*
the other bands, i.e. the diazones of PREISWERK (1895) have a minimal effect on the light passing through them ; they transmit fully at all visible wavelengths and, therefore,appear "neutral".

7.4. certainly increased when a ground section is examined in plane polarised light (polariser only); it is maximal when the vibration direction (electric vector) of the incident radiation is approximately parallel to the unique axes of the ordered elements (i.e. the long axes of the prisms and crystallites).

SCOTT and WYCKOFF (1947) showed that the HUNTER-SCHREGER bands could be made visible in replicas of the polished surface of a longitudinal section of human enamel as a result of differential etching of the different "zones" - presumably (in my opinion) only related to a different orientation of the structural elements in the surface. MARTELL and PEYTON (1956) repeated this work with the same results and concluded that the mineralisation is such that alternating bands show greater and lesser resistance to etching. If we are generous enough to include a consideration of the orientation of the mineral component under the general heading "mineralisation", then we may suppose that these authors are correct.

NEUBRUN (1962) and AVERY (1963) found variations in the (micro) hardness of the enamel corresponding with the architectural arrangement of the enamel prisms, confirming similar results reported by BURG (1921).

The first workers to observe the HUNTER -SCHREGER bands in microradiographic images of ground sections assumed that these bands showed a real difference in mineral content (HOLLANDER, BÖDECKER, APPLEBAUM and SAPER, 1935). STAZ (1946) thought that the HUNTER-SCHREGER bands "may indicate stages in calcification of enamel if recent views on amelogenesis are accepted" (i.e. DIAMOND's and WEINMANN's (1940) view that enamel maturation proceeds from occlusal to cervical at right angles to the incremental lines). RÖCKERT (1955) thought that - "these bands indicate that there is a rhythmical calcification and that their appearance is not due to an optical phenomenon". BAUD and HELD (1956) correlated their microradiographic finding of a low x-ray absorption in alternate HUNTER -SCHREGER bands with an increase in the amount of silver deposited after immersion in silver nitrate solution.

7.5.

DARLING and CRABB (1956) found "variations in calcification, apparently corresponding to the bands of SCHREGER" in only two out of one hundred sections which they examined by microradiography.

AMPRINO and CAMANNI (1956) found that the HUNTER-SCHREGER bands "are not always detectable" in microradiographs (they show well in their Figure 1A of dog enamel) and considered that (p.224) -" it is doubtful whether they represent areas in which the microscopic enamel constituents show differences in their calcification". They also attempted an explanation of this phenomenon, based on the orientation of the prisms in the HUNTER -SCHREGER bands, stating that (p.245)" As the orientation of prism bundles is different in contiguous Schreger's bands, the x-ray absorption should vary therefore according to whether the prisms are cut perpendicularly or parallel to their length". In a footnote (p.245) they wrote -" In a given volume of enamel, the ratio between the amount of less calcified interprismatic substance and highly calcified prism matrix proper should obviously be different in these conditions; the difference should be more apparent the thinner the tooth section. In fact, Schreger bands are more easily detectable in historadiographs of thin sections". GUSTAFSON and GUSTAFSON (1961) considered that the HUNTER -SCHREGER bands showed up because of differences in the direction of the prisms, "Which allow the x-rays to penetrate more easily along and between the cross-cut prisms".

The observation (SCOTT,NYLEN and PUGH, 1962) that the alternate layers (i.e. rows; lamellae; "Pseudoprismen" of KORVENKONTIO, 1934-35) of prisms in mouse incisor enamel are revealed in the contact microradiographic image will probably have removed the doubt which may have existed in the minds of some workers that a difference in orientation of the prisms can, in some way,occasion a variation in contrast of the developed (silver) photographic image. It is not possible that there can be any difference in the mass of mineral per unit volume between regions of enamel containing right or left handed zones. Even if "interprismatic substance" were less calcified it would still be contained in the same proportion in any significant portion of enamel.

7.6. The most likely explanation* of this peculiar phenomenon is that variations in the section thickness determine the variations in x-ray absorption in ground-section specimens. The different orientation of crystallites in the different zones (HUNTER-SCHREGER bands) will determine that they are abraded (probably fractured out of the surface) at different rates. FEARNHEAD (personal communication) has shown that the HUNTER-SCHREGER bands are particularly prominent in microradiographs of sections prepared with the "airbrasive" technique. ANGMAR, CARLSTRÖM and GLAS (1963) make no mention of the appearance of HUNTER-SCHREGER bands in contact microradiographs of ten (selected as) plane-parallel longitudinal ground sections of human teeth.

The most important comparative anatomical studies of prism-decussation are those of TOMES (1850 - Rodentia and Lagomorpha), PREISWERK (1895), KORVENKONTIO (1934-35 - Rodentia and Lagomorpha), and KAWAI (1955). The narrowest zones occur in the Rodentia (1 prism wide in the Myomorpha and Scuiriomorpha) and the widest in the Ungulata . There is no decussation in the Odontoceti, Cheiroptera, Insectivora and Sirenia .(For further details see BOYDE, 1964 MS).

PREISWERK (1895, 1903) introduced the term "zonies"for referring to groups (rows, lamellae) of prisms running in the same direction. Groups of predominantly transversely sectioned prisms were called Diazones and longitudinally sectioned groups, Parazones. PREISWERK's (1895) dia- and para-zones were present in a "middle layer" and absent in "basal" - and"superficial-layers".

The decussation of alternate transverse rows of prisms in murine incisor inner-enamel has also been recognised by ERDL (1841), OWEN(1845), ROLLESTON (1871), von EBNER (1890), TOMES (1904), ADDISON and APPLETON (1915),LAMS(1921),ORBAN(1925B), PINDBORG(1950),BUTCHER and TAYLOR(1955), BUTCHER(1956), HELMCKE and RAU(1962),BOUYSSOU,GUILHEM and VIALLE(1962), BOUYSSOU(1963), HELMCKE(1963 A,B) and many others. In spite of the wide recognition of this pattern, it has gone unheeded - with consequent serious errors of interpretation by numerous authors (details BOYDE,1964 MS).

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* OSBORN, 1964,has reached a similar conclusion.

7.7. RASCHKOW and PURKYNĚ (transl. in NASMYTH , 1839, A. p.144) realised that the decussation of enamel prisms implied a relative movement of ameloblasts (or groups of ameloblasts)past each other (if one ameloblast is related to one prism throughout the entire thickness of the enamel). Acceptance of this movement of rows of cells past each other has proved too much for some authors and they have been led to deny or at least question its existence(WILLIAMS, (1896), GOTTLIEB,(1943), AGNEW, (1947)). A few authors have described changes in orientation of groups of ameloblasts (as appreciated in a longitudinal section of the developing tooth) which they have ascribed to this relative movement of ameloblasts (JASSWOIN, 1925 - also cited von EBNER in this respect ; MEYER, 1935). ORBAN (1925 B) stated that the "enamel of rat incisors consists of ellipsoidal prisms which cross each other at right angles and whose formation proceeds through the ameloblasts crossing each other" and claimed that his Fig. 25 (p.620) showed this crossing of the ameloblasts at their nuclear-ends. He seemed to have completely forgotten this earlier work, when in 1943 he subscribed to a radically different interpretation of the same histological picture.(ORBAN,SICHER and WEINMANN (1943 A,B) wrote of a rhythmical transformation of the rods to form the cross striations of the mature rods in rat incisor enamel). HELD (1926) probably recognised the decussation pattern for he talked (in caption to Fig. 7 of Plate 8) of "every alternate row cut transversely"("je eine Reihe quer getroffen"). SYMONS (1957) has figured clearly defined rows of transversely sectioned ameloblasts in tangential sections of the developing rat incisor. BUTCHER and TAYLOR (1955) and BUTCHER (1956) considered that the ameloblasts of the rat incisor,"must pass or shift by the ameloblasts in adjacent layers" - BUTCHER thought this to be -"phenomenal, yet not impossible". ADDISON and APPLETON (1915) and LAMS (1921) studied the development of the rat incisor, and apparently noted nothing peculiar in the derivation of the decussated prism pattern (which they correctly described) from the parallel cells of the ameloblast layers.

There has been remarkably little speculation by previous workers on the nature of the factors initiating and maintaining decussation.

7.8. WALDEYER(1870) held that -"we may reasonably refer many of the peculiarities in the course of the enamel prisms, especially their decussation, spiral course, and undulations, as well as their optical characteristics" -." to the papillary projections of the dental sacculus" (i.e. to the organisation of the remaining cell layers of the enamel organ, and particularly the arrangement of the capillaries).

SÜSS (1940)presupposed the existence of rows of ameloblasts which maintained contact with each other at their outer ends - these rows (5-8ameloblasts or prisms wide in dog) being successive generations of ameloblasts. He supposed that an increase in the surface area of the dentine (presumptive enamel-dentine junction or basement membrane, one must suppose) occurred at the commencement of dentinogenesis, whereas an equivalent increase in the surface area of the outer ends of the ameloblasts could not occur because they were held together in some way (perhaps he was thinking of the terminal bar apparatus, although no mention is made). The difference in the surface area of the inner ends of the ameloblasts compared with their outer ends was supposed to start a screw motion of their inner ends, which developed into the sliding motion of the rows.

WOLF's (1942) concept is no easier to follow. He held that (loc. cit. Conclusion 3) that "the cause of the sliding of the ameloblasts and the concomitant slanting growth of the prisms lies in the necessity for the ameloblast layer to accommodate (or adjust) for the growth in breadth (? of the tooth) at the expense of height.(Conclusion 4) This adjustment to the greater breadth at the cost of height can only be accomplished - given a constant number of ameloblasts of constant dimensions - through a turning about in rows (Umreihung), which rows glide next to one another".

Decussation of the secretory territories of ameloblasts (and hence the prisms) may be caused, controlled and/or maintained by factors resident either: 1)in the ameloblasts themselves, or: 2) in their secretory territories.

The inner ends of the ameloblasts in the rat incisor (inner-enamel) cross each other up to a level several microns towards the nucleus

Figures 7.1 and 7.2

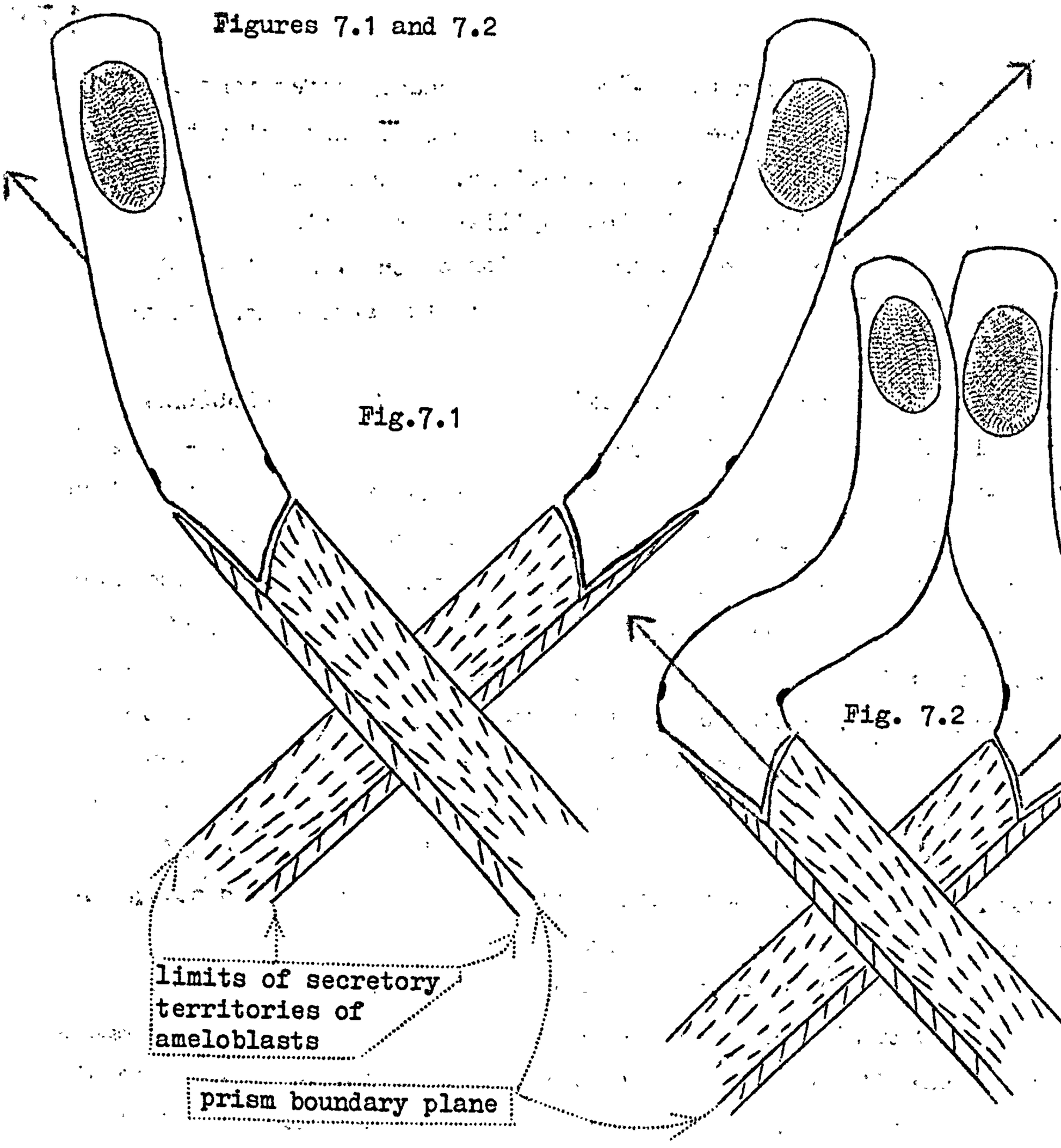


Fig.7.1

Fig. 7.2

Does the movement of the ameloblasts (fig. 7.1) or the mode of growth in the walls of the depressions in the mineralising front in which the ameloblasts are anchored (fig. 7.2) occasion the relative sliding movements of ameloblasts past each other which must occur during the development of decussated prisms?

It would seem more probable that the ameloblasts would lead the prisms were the first alternative correct: in which case the prisms would lie in much the same straight line as the long axes of the ameloblasts (which is not the observed case) and the join between prisms and ameloblasts would be a smooth, single curve.

If the eccentric growth of the depressions in the mineralising front led the ameloblasts it would be expected that the prisms and ameloblasts would lie at an angle to each other,(which is the observed state of affairs during the development of decussating prisms) and that the ameloblasts would show a double kink just outside the surface of the developing enamel. (see Fig. 7.3.)

7.9. away from the inner terminal bars, whereas the greater part of the length of these cells is parallel . It should, therefore, be possible to determine whether factors 1) or 2) are operative by making an accurate reconstruction of the "angle" between the prisms and the ameloblasts.

1) If the ameloblasts initiate and maintain decussation by an active movement on their part, the "angle" formed between (the "depression in the mineralising front" containing) TOMES' process, and the inner end of the relevant ameloblast would be a smooth curve since the ameloblast is "leading" its own secretory territory.

2) Alternatively, the "secretory territories" (depressions in the mineralising front) may maintain decussation by eccentric growth, i.e. by the deposition of more material on one side of each depression. (This may be controlled by the ameloblasts, i.e. they may be able to secrete selectively on one side of TOMES' process, or it may be a result of the mode of growth of the depressions themselves). Assuming that the TOMES' processes are effectively anchored in their own depressions - the "filling in" of these depressions from alternate sides would drag the alternate rows of ameloblasts in opposite direction. In this case there would be a double bend or kink between the TOMES' process and the remainder of the cell (diagram Figure 7.2.). In the absence of any direct experimental evidence I would favour the view that the decussation of the prisms is a result of the eccentric deposition of new material in the walls of the secretory territories of the ameloblasts. My reasons for supporting this view are: a) that there are no other recorded examples of the relative horizontal movement of member cells within a sheet of columnar epithelium ; and b) that since prisms are essentially groups of groups of crystallites - and there are grounds for believing that groups of crystallites, once formed, tend to go on growing in the same direction as a group (2.5.) - their innate tendency to keep growing in their own direction would explain how the secretory territories "grow" eccentrically; "dragging" the ameloblasts after them. The rapid parallel growth of groups of crystallites might outstrip the possibility for new growth of groups at any other angle, and in addition the "stroking" orientation effect (postulated in Chap.2) would tend to perpetuate a situation in which

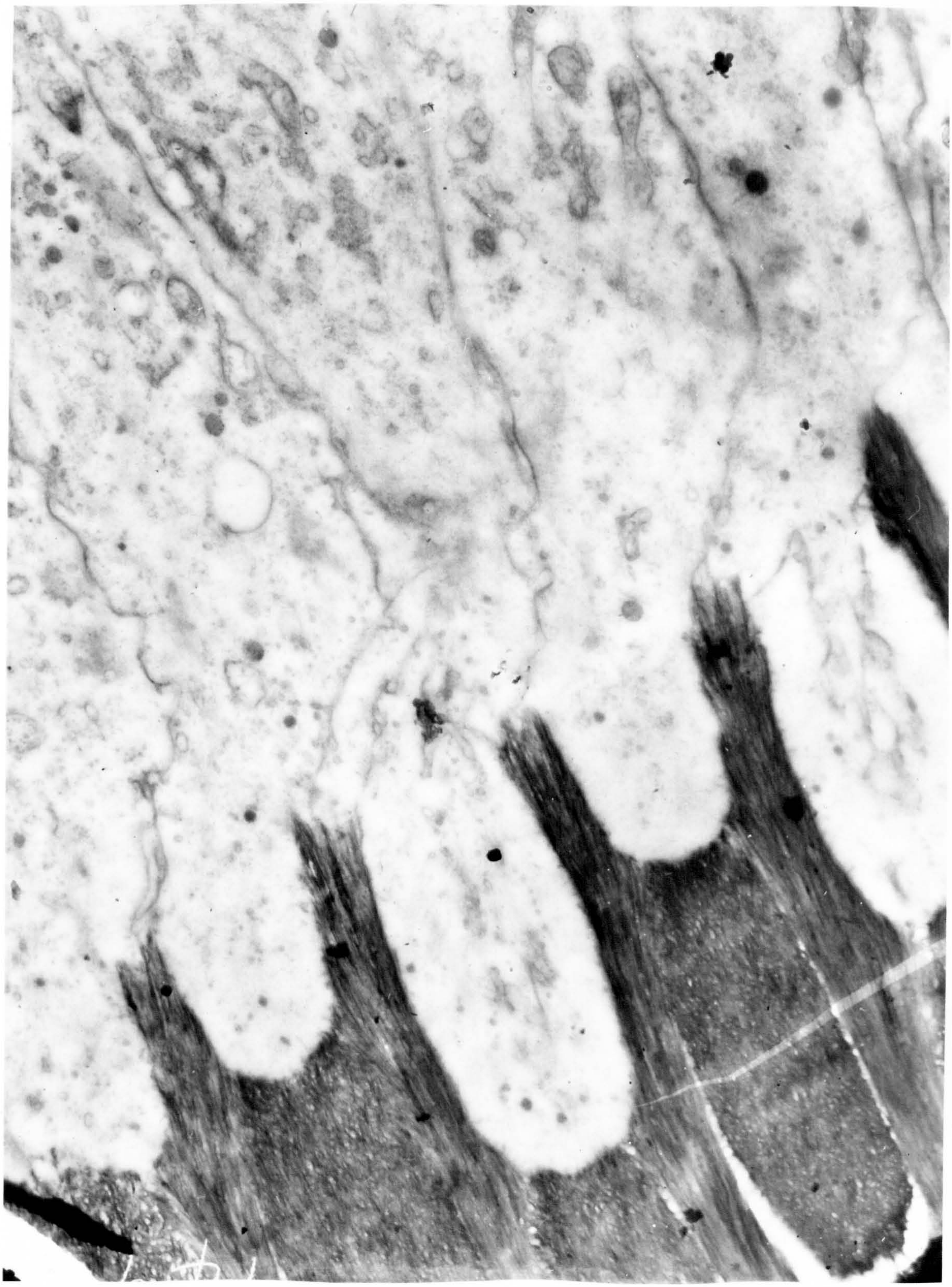


Fig. 7.3.

Figure 7.3. Rhesus macacus: T.S. tooth. (X 13000)

7.10. decussation had already begun.

Other factors that might be involved in determining that a "prism" continues to fill-in from a given side are:-

1) that the sides of the mineralising front which are contained in the angle between the prism and ameloblast axes (i.e. the sides in which the intradepression crystallites develop) have the largest surface areas. This would mean that the chances for the deposition of new material in these regions of the mineralising front might be greater.

2) The ameloblasts in some species may be able to secrete from one side of their TOMES' processes. It is not definite that all the surfaces of TOMES' process have the same structure, equivalence or potentiality. The sides of TOMES' processes adjacent to the circumdepression crystallites (2.5.) move relative to the mineralising front. This may determine that the conditions for crystallite growth are relatively poor in these regions of the mineralising front, or simply that there is a reduced chance that the "secretion" elaborated by the ameloblasts will be shed from these surfaces of the TOMES' processes.

3) It is possible that the mode of attachment of ameloblasts to each other may not be the same all round each cell at the level of the terminal bars or elsewhere. If the ameloblasts of one zone (single row in the Muridae and Sciuridae) proved to be attached to each other more firmly than to the cells in adjacent rows, this could explain the great regularity of the decussation of the prisms.

The present study has not revealed any fundamental differences between the formation of enamels with "round" (PATTERN 1: Fig. 1.1.) prisms which do not decussate and those enamels with "horseshoe" (PATTERN 2: Fig. 1.2.) or "arcade" (PATTERN 3: Fig. 1.3.) prisms which do decussate: but it is possible that the difference may be simply that the ameloblasts never lie at an angle to the prisms during the development of PATTERN 1 enamels, so that the trend to eccentric growth in the depressions in the mineralising front never gets a chance to get under way. The preservation of the manatee (Sirenia : PATTERN 1 enamel) ameloblasts was very poor so that it is not yet possible to say that

Figure 7.4.

Photograph of page 241 of the Journal of Ultrastructure Research Volume 6, i.e. Figure 8 of Ronnholm's paper "An Electron Microscopic Study of Amelogenesis in Human Teeth 1 . The Fine Structure of the Ameloblasts".

The double kink in the ameloblasts and the direction of their long axes with respect to the prism direction imitates exactly the arrangement postulated in Figure 7.2. This is held to indicate that the unequal deposition of new material in the walls of the depressions in the mineralising front of the developing enamel is the prime factor in determining the prism direction; and that it is this "force" and not an active movement of the ameloblasts which causes the decussation of the prisms.

FIG. 8. Survey picture of the ameloblasts and the enamel prisms. The boundary between the ameloblasts and the enamel seems serrated in longitudinal sections. Each tooth in the saw-like serration is contributed to by one ameloblast cell. × 7700.

Figure 7.4 RONNHOLM's (1962A) Figure 8

Fig. 7.4.

11. there are no obvious differences in the terminal bar apparatus of ameloblasts which slide past each other, and those which do not.

The most important general implication of the presence of terminal bars in cells which slide past each other lies in the fact that this movement is permitted. This has not been commented on previously. It means that we must not conceive of the terminal bars as "cementing" the cells together - the nature of the forces attracting the terminal bar region, cell membrane surfaces must be analogous to those of the surface tension holding two wet glass plates together - though they are, naturally, quite certainly not of the same physical nature.

The demonstration that zone formation - the decussation of alternately directed layers of prisms - begins as a spiral formation in the region of the "gnarled" enamel over cusps (KAWAI, 1953: see section 5.3.) may help to explain how these zones are initiated. Aside from a complete shift of the whole ameloblast layer, such as that occlusal shift which accompanies the development of the occlusal slope of the prisms, all movements of individual ameloblasts* or groups of ameloblasts in one layer must be matched by the movement of an equivalent bulk of ameloblast-substance through an equivalent distance. It is only necessary for one group of ameloblasts to commence to glide past their neighbours and the whole process or zone-formation may have been initiated. There is an increased likelihood of a group of ameloblasts making an angle with their prisms during the first stage of deposition of enamel over the tip of a cusp, since the surface of the developing enamel is at its most convex. A sliding movement which occurred near the tip of a cusp might well result in a spiralling of this movement - matched equally by an opposite movement of an adjacent and equal number of ameloblasts - down around the cusp tip. This initiation of zone formation need not then

*The factors which might control the position of the ameloblasts at intervals of time during their secretory life-span are: 1) their inner ends are anchored in the secretion with which they have surrounded themselves, 2) They are attached to each other at both ends by terminal bars, 3)They are probably guided in their outwards path by the very manner in which they are closely packed together.

7.12. continue as a spiral once the zones reach a certain size. However, the fact that decussation does occur in enamels which only cover one tooth surface (e.g. in the rodent incisor) would suggest that other more complex (zone - formation) initiating factors perhaps inherent in the ameloblasts themselves must be invoked.

GUSTAFSON (1945) proposed the existence of supplementary HUNTER - SCHREGER bands -"wedged in"- where the external surface of the tooth curves markedly: -"The ramifications which have been observed in the Hunter - Sohreger lines are caused by supplementary shorter Hunter - Schreger lines which do not run all round the tooth as do the regular lines". The existence of "supplementary" bands is easily confirmed by examining the arrangement of the HUNTER-SCHREGER bands as seen through the surface of the enamel in intact teeth in the Carnivora and Hystricomorph rodents.

CONCLUSIONS. (CHAPTER 7)

suppl 7.12. (1) The so-called HUNTER-SCHREGER bands were described adequately many times before HUNTER (1770) and SCHREGER (1800) forwarded their descriptions. Both on these grounds and because this name does not suggest the underlying nature of the arrangement of the prisms it is felt that the terms "prism decussation" and "zones" (after PREISWERK, 1895) should be more widely adopted.

(2) Prism decussation is caused by the unequal growth of the walls of the depressions in the mineralising front in the developing enamel. The depressions fill in from alternate sides in alternate zones and "lead" the inner-ends of the ameloblasts after them.

(3) The one-sided filling in of the depressions in the mineralising front (which determines prism direction and decussation) is maintained by dynamic factors related to the relative movement between the surface of the TOMES' process and the mineralising front: the conditions for crystallite growth are relatively poor in those faces of the depressions in the mineralising front where there is a relative sliding movement (see Fig. 2.34).

CHAPTER V111

STRATIFICATION IN MAMMALIAN ENAMEL.

(DISCUSSION).

8.1. Incremental lines in enamel.

The so-called brown striae of RETZIUS (1836,1837) have always been accepted as incremental lines (PURKYNE and FRAENKEL, 1835; LINDERER and LINDERER, 1837). Their relationship to the Perikymata (PREISWERK, 1895; circular wrinkles of LEEUWENHOEK, 1678) was noted by RETZIUS (1837), PICKERILL (1913),PANTKE (1957) and others: it implies that the Perikymata are also related to the pattern of growth of the enamel. The evidence that the "brown striae" mirror stages in growth is that they parallel the successive stages of deposition of layers of enamel as this has been observed on countless occasions. The Perikymata are approximately parallel to the growing edge of the enamel cap; and hence, presumably, to the part of the enamel cap which has just ceased growth at any one time.

The brown striae are "normal" structures (MELLANBY,1927) - their etiology is unknown. Their development has never been visualised in a histological study. RUSHTON (1933) is one of many who could find no correlation between possible causative factors and the pattern of incremental lines in human deciduous teeth. However, their disposition in individual teeth and the fact that the same pattern of incremental lines forms in all the enamel forming at one time (FUJITA,1939; A-G.

8.2. GUSTAFSON, 1955) make it certain that their formation is controlled by systemic factors rather than local-environmental factors.

RETZIUS (1837) himself proposed that the incremental striae were in part caused by a pigment and in part by a bending of the prisms. It has also been proposed that they may be both more or less mineralised (A-G.GUSTAFSON, 1959); that they are less mineralised (BAUME, 1882; KEIL, 1936; LEHNER and PLENK, 1936; RÖCKERT , 1955; BAUD and HELD,1956); and that there may be a variation in the proportion of "interprismatic substance" or in the width of the "organic prism sheath" associated with them (von EBNER, 1906; MEYER, 1925, 1935; A-G.GUSTAFSON,1959;SOGNNAES, 1949; JANSEN and VISSER, 1950). The pigmentation theory has held wide support (e.g. HERTZ, 1866; WILLIAMS, 1896,1923 A; TOMES, 1904). Associated bends in the prisms were noted by KOLLMAN (1871), GUSTAFSON (1945), SCHMIDT and KEIL (1958) and A-G.GUSTAFSON (1959). RÖCKERT (1955) and BAUD and HELD (1956) reported the appearance of RETZIUS ' lines in microradiographs of normal enamel. It is well known that the prominence of the brown striae may be accentuated in silver "stained" (in $AgNO_3$ solutions) ground sections : BAUD and HELD (1956) believed that this indicated a low degree of mineralisation in the striae. Many other workers have held that the brown striae reflect a mineralisation rhythm in the enamel (FUJITA, 1939; SCHOUR and HOFFMAN, 1939 A).

CZERMAK (1850) proposed that the "underlying cause of the behaviour of these stripes against the light", might be the presence of spaces. KOLLMAN (1871) thought that the striae of RETZIUS were not pigmented, because their appearance changed with the direction of illumination. von EBNER (1890) noted that their colour is a blue-white in reflected white light, which is complementary to the brown seen in transmitted light, and he concluded that their colour was due to an "interference" effect. von EBNER considered that the brown striae are more clearly visible in sections of dried teeth (confirmed by ASPER, 1916 - denied by TOMES, 1904 and WILLIAMS, 1896 A): also that they could be removed by boiling for several hours when - "nothing remains of the stripe but a bright line". His laboured considerations about the mode of entry of air into cracks in the interprismatic cementing substance (Kittsubstanz") may seem rather facile today, but he was certainly proceeding along the right lines when he wrote (1890, p.65) "The brown colour of the parallel

8.3. striae originates from air which is situated in cracks (Spalten) between the enamel prisms". PICKERILL (1913, p. 972) noted that - "the Striae appear an intense white by reflected light, this may be imitated exactly by painting overlapping films of Chinese White (zinc oxide) on a glass slide, ... when the overlaps appear brown by transmitted, but white by reflected light".

KEIL (1936) found that the RETZIUS lines remained dark in all positions between crossed nicols - "As a result of their poor calcification". He found that they sometimes showed an anomalous behaviour in that they appeared bright when the rest of the enamel was dark. He concluded that there was an "orientation of crystals in this direction" and (from his figures 11, A and B, text and captions) it would appear that this orientation of the crystallites in the Striae of RETZIUS is about 40° to the prism direction (the outer ends deviating cervically). A-G.GUSTAFSON (1959) distinguished between functional RETZIUS lines in which there were bends in the prisms, and pathological RETZIUS lines, which were associated with an increased amount of inter-prismatic substance; either hyper- or hypo-mineralisation of "prism segments"; prominent "intersection sheaths" (? cross striations); or compression of "sections" - (i.e. a narrower cross-striation repeat interval). The evidence that they are associated with differences in the degree of mineralisation is not acceptable.

The foregoing brief review shows that the most distinctive features of the Brown Striae are: 1) their colour: 2) their association with bends in the prisms or: 3) changes in the width of the "interprismatic substance" or 4) a reduction in the cross-striation repeat interval. The changes in the crystallite orientation pattern implied in features 2, 3 and 4 above, all suggest that there is a change in the rate of secretion of the enamel at the time that the striae are formed - this would lead to a change in shape of the mineralising front and hence to significant changes in the crystallite orientation pattern (viz. "bends" in the "prisms" and changes in the proportion of "prism" to "inter--prismatic substance"). Features 3 and 4 suggest that the rate of secretion is reduced (not increased). Since the cross-striation repeat interval represents the daily growth increment (ASPER, 1916; KOMAI,1942), a reduced interval obviously implies a reduced growth rate. Not so

8.4. obviously, however, an overall reduction in growth rate might lead to an increase in the proportion of "interprismatic substance" (circum-depression crystallites) to prism substance. A reduction in the rate of secretion from the ameloblasts would lead to a reduction in the rate of the sliding movement of TOMES' process past the circum-depression crystallites (see Chap.2 for terminology). The accompanying reduction in the "stroking" crystallite orienting factor would allow a greater divergence of the circum-depression crystallites from the "prism direction" (i.e. direction of progress of the ameloblasts): if the crystallites in intra-depression and circum-depression situations still continued to grow at the same relative rates then an increase in the width of the inter-prismatic regions (PATTERN 1), interrow sheets (PATTERN 2) or winged process regions (PATTERN 3) would be forecast (Diagram.8.1.). The crystallites in developing enamel can always be found close to the ameloblasts. A reduction in the rate of growth of "enamel" therefore implies a reduction in the rate of growth of (the long axes of) the apatite crystallites. A reduction in the overall rate of deposition of crystalline material would be expected to favour the growth of fewer, larger crystals. This statement is justified by the well known principle in growing single crystals in solution of limiting the rate of deposition (by, for example, limiting the ion concentration in solution) and all the possible disturbances - a reduced rate of secretion might be expected to reduce the "turbulence" (FEARNHEAD, 1961B) in the vicinity of the developing front . The presence of fewer, larger crystals separated by fewer, larger spaces (i.e. a greater inhomogeneity) in the "brown" striae of RETZIUS would explain their"colour".

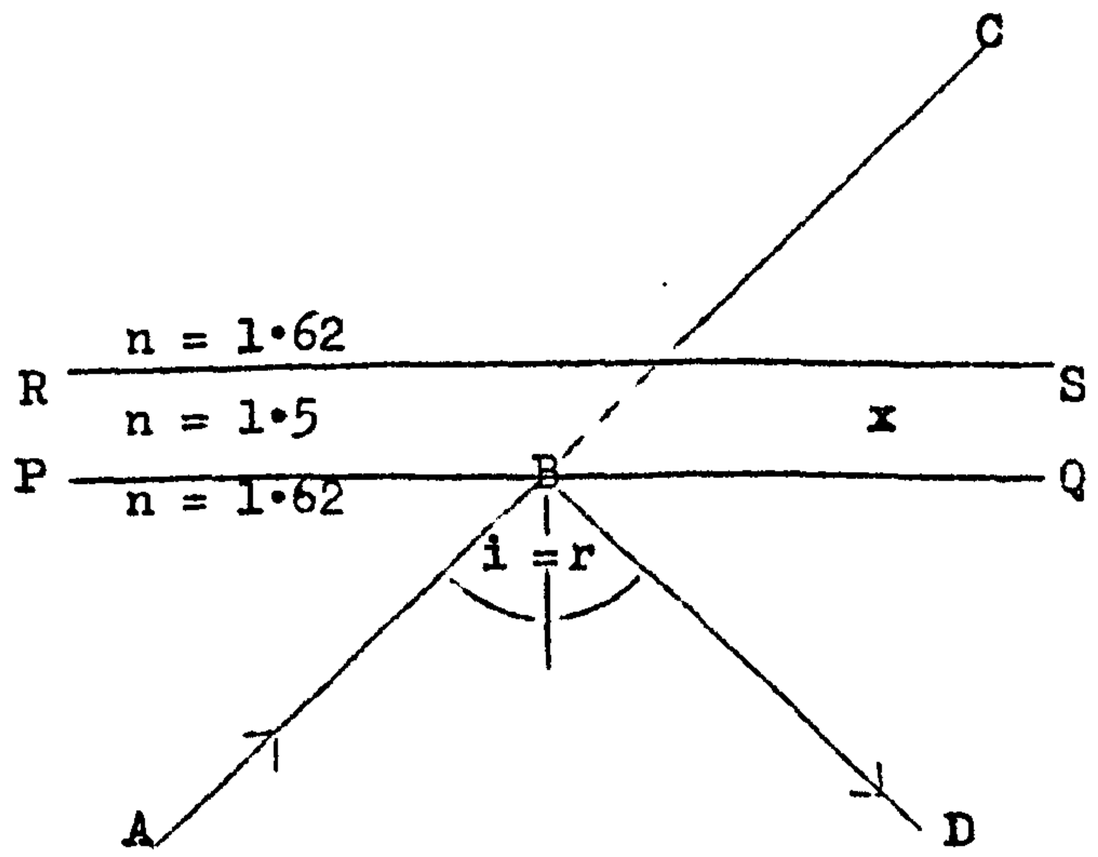
The fact that the colour of the "brown" striae is very dependent on the direction of illumination makes it certain that it depends on some difference in structure from the surrounding "normal" enamel. The "brown striae" scatter predominantly blue light from a polychromatic source; longer wavelengths are transmitted and the striae,therefore,appear yellow or orange in colour by transmitted light. It is not possible to state whether the transmission loss of the shorter wavelength radiation is entirely due to scattering(by "particles" much smaller than the wavelength of light), because multiple internal reflection (from plane interfaces

3.5. of semi-infinite extent in the plane of the interface; but only separated from an adjacent interface by a gap much smaller than the wavelength of light) could produce a similar effect.

The transmission loss (in the visible spectrum) from one reflection at one double-interface in an otherwise homogeneous medium is inversely proportional to the wavelength of the incident radiation. RAYLEIGH scattering (from separate, spherical particles, denser than the surrounding medium; of radius r where r is small in comparison to λ) is proportional to $\frac{1}{\lambda^4}$, and is, therefore, extremely dependent on the wavelength of the incident radiation. RAYLEIGH scattering is also proportional to r^6 : it would therefore be imagined that small increases in the crystallite size in the enamel would result in a great increase in scattering, particularly of the shorter wavelengths; and that scattering is the predominant mechanism in the formation of the "colour" in the "brown" striae. If a ground section is illuminated with white light at an angle of incidence approaching the normal to the direction of observation, the blue scattered radiation is polarised; with the vibration direction (electric vector) normal to the direction of the original beam: this evidence also suggests that scattering occurs.

The underlying structural cause of the enhanced scattering in the brown striae must be the presence of larger crystallites or larger spaces between the crystallites. It is not necessary to assume, or possible to deduce, that there is a change in the quantity of the mineral component in the striae - the same amount of mineral could be present as fewer, larger crystals; separated by wider spaces containing less dense, lower refractive index materials, e.g. organic matter, water; and air in dry, ground sections. The colour of the "brown" striae is enhanced in dried ground sections; and disappears completely when the spaces are imbibed with a medium having a refractive index close to that of enamel (n =1·62).

8.6. The accompanying diagram should be consulted if it is not immediately evident that wavelength selective reflection can depend on the size of the intercrystalline spaces in enamel. Light incident on a boundary (PQ) between two media (n = 1·62 and n = 1·5 say) will be totally internally reflected (path ABD) in the higher refractive index medium - in this case "enamel" (i.e. a "homogeneous" mixture of enamel crystallites and organic matrix in which the intercrystalline spaces are too small to be considered as inhomogeneities - they are below a certain



fraction of λ). If another similar boundary (RS) approaches PQ, - the separation between PQ and RS being x - then at a particular value of x, say x_1, a significant fraction of the incident light (AB) will continue along its original path (ABC) instead of being reflected (ABD). The value x_1 must be a certain fraction of λ. Longer wavelength radiation will, therefore, proceed along ABC before the shorter wavelengths.

BERGGREN (1947 p.39) noted that "The Striae of RETZIUS have such an affinity for methylene blue that not only do they become stained themselves but they act as a barrier to further penetration". This observation would tend to confirm the present hypothesis - that there are larger spaces in these regions and also shows that these spaces are more permeable. GUZMAN, MERMAGEN and BRUDEVOLD (1955), G.GUSTAFSON (1957A), DARLING (1958) and many others have noted that the Striae of RETZIUS become selectively demineralised during the carious attack on human

8.7. enamel. DARLING (1958) held that the "cariogenic agent" entered the enamel through the surface zone along the Striae of RETZIUS and that the latter seemed to aid the spread of the lesion. The selective spread of the carious lesion along the Striae of RETZIUS would be aided both by the initially larger size of the permeable spaces between the crystallites in these regions and by the fact that there would be a proportionally larger increase in the size of these spaces after the dissolution of a given mass of enamel mineral ; as compared with the removal of the same mass of mineral from the surface of a larger number of smaller, more closely positioned crystallites in the adjacent "normal" enamel.

Any explanation of the development of the striae must account for the fact that the fundamental, initiating change in the nature of the enamel in these regions occurs at the time that it is laid down and yet the amount of the mineral component increases and the organic component decreases for a considerable time afterwards(during maturation). The controlling factor is systemic and could be of the nature of a hormonal imbalance or general systemic "poisoning" of some sort. Mineral, vitamin, or other nutritional factor deficiencies or excesses, can be excluded on the grounds of the universal occurrence of the striae.

The evidence that the changes in structure in the striae (whatever their nature) are not initiated within the already formed and maturing enamel at some distance from the mineralising front lies in the arguments just presented (viz: that the rate of growth of the enamel is reduced) that it is the enamel with narrower cross-striations which is actually in the striae (A-G. GUSTAFSON, 1959). The view of maturation presented in this thesis would suggest that the final mineral and organic contents are determined by the growth of the mineral component, which means that concepts of the formation of the striae of RETZIUS involving considerations of changes in the organic:inorganic ratio (or the total amount of organic matrix in the initially secreted "enamel") become absolutely meaningless. If the formation of the brown striae is determined by the organic "matrix" then it must be due to a fundamental change in its nature - either it would have to be much more resistant to being removed again during maturation - or, if we admit of its fibrous nature and that mineralisation occurs by epitaxy (RÖNNHOLM, 1962; FRANK and NALBANDIAN, 1963) on the fibres, the "fibres" must be fewer and

8.8. farther between, in order to account for the increased separation between the crystals. A change in the organic matrix of either of the two natures just proposed is improbable: it is more likely that change in the organic component is secondary to change in the mineral component and that this in turn is associated with a general slowing down of the rate of secretion.

Until it can be shown (with a reliable analytical method) that there are real differences between the mineral content of the incremental lines and the surrounding enamel, it must be assumed that they appear in the light microscope image because of differences in the crystallite orientation "pattern" [viz:- 1)bends in the prisms : 2) changes in the proportion of prism and interprismatic regions: 3) the change in crystallite orientation observed directly by KEIL (1936) : and 4) the more and less negatively birefringent prism segments of A-G.GUSTAFSON (1959)] and because there are fewer, larger crystallites with larger spaces between them. It cannot be excluded that there is a change in the organic matrix which controls the last factor, e.g. a reduction in the total number of nucleating sites at the mineralising front. The view of the nature of the changes associated with the development of the brown striae of RETZIUS that I favour is that a reduced rate of growth of the enamel is associated with a reduced rate of deposition of "mineral", and that what "mineral" is available is used in growing fewer, larger crystals. This concept would be contradicted by the positive demon-stration of a protein fibre epitactic nucleation mechanism in enamel.

Rate of Formation. SCHOUR and SMITH (1934) found that the enamel of the rat incisor was deposited continuously at a rate of 16 μ per day ; SCHOUR and STEADMAN (1935) confirmed these results and SCHOUR and HOFFMAN (1935 - 36 B) found the same rate of apposition of enamel - viz. 16 μ per day - in rats, ground squirrels, guinea-pigs, rabbits, kittens and puppies. SCHOUR and HOFFMAN (1935 - 36 A) described pairs of light and dark (incremental) lines at 16 μ repeat-intervals in the enamel of various tooth types in the opossum, rat, ground squirrel, guinea-pig, cat, dog, macaque monkey and man. SCHOUR and PONCHER (1937) found the average rate of apposition of the gingival enamel of the lower second deciduous molar of a 5 months old human infant to be

8.9. 3.92 μ per day. SCHOUR and HOFFMAN (1939 A) again published their observations of a 16 μ "stratification" (calcification) rhythm; but this time they embraced the enamel and dentine of animals from fish to man! These observations have never been confirmed, and there is little doubt that the magical figure of 16 μ would best be forgotten (see BOYDE, 1964 MS; SCHOUR and HOFFMAN, 1939 B; SCHOUR, 1937; MASSLER and SCHOUR , 1946).

KORVENKONTIO (1934 - 5) cited various earlier workers as providing estimates of 400 - 500 μ per day for the rates of eruption, attrition and growth of the lower incisors of the rat. Since one ameloblast is approximately 4 - 5 μ in diameter, 100 new rows of ameloblasts are formed per day ! PINDBORG and WEINMANN (1959) estimated that the inner layer of the rat incisor is completed in 6 days and the outer layer (which they called "fibrous"; after TOMES, 1850) in a further 3 days. These figures for the rate of formation of the enamel agree well with those published by SCHOUR, but their derivation is not given.

8.2. The cross-striations of the prisms. The lack of direct developmental (histological) evidence has proved no barrier to speculation based on the observed structure of the cross-striations. WALDEYER (1865) thought that they were caused by the decussation of the prisms ; in the same way as the false cross-striations in murine incisor inner-enamel (ERDL, 1841). HERTZ (1866) and WENZEL (1868) pointed out that this would confine the possession of cross-striations to the prisms at the periphery of the decussating "zones" (PREISWERK, 1895). Most workers have held that the cross-striations mirror in some way the deposition of successive increments of enamel (PURKYNĚ and FRAENKEL, 1835; RETZIUS, 1837; SCHWANN, 1839; TOMES, 1848; CZERMAK, 1850; HANNOVER, 1855; BAUME, 1882; and many others). Opinions have differed widely as to whether they are formed by the deposition of discrete segments (ANDREWS, 1891; WILLIAMS, 1896A; SCHOUR and MASSLER, 1940), or whether they reflect cyclical changes in the transformation of the ameloblasts or in the composition of the material secreted by the ameloblasts, so that the prisms have a corresponding or correlated variation in composition

8.10. along their length; leading to a change in refractive index and hence their visibility with the ordinary light microscope (DARLING,1958; GUSTAFSON,-1957B; GUSTAFSON, 1945). WILLIAMS,(1895) thought (like LINDERER and LINDERER,1837) that the varicosities might be caused by a set of "cross fibres". He thought this to be more likely than - "that there may be a rhythmic simultaneous action of all the ameloblasts concerned in the deposition of the matrix for enamel building".

Most workers who have concerned themselves with the organic matrix in sections of decalcified enamel have described the "septae" of organic material outlining the cross-striations as confined inside the prism-sheath. WEIDENREICH (1926 - holder of many opinions diverging the "norm") thought that the cross-striations involved the whole organic matrix: this he saw as evidence in favour of his theory of the purely physical nature of the process of calcification in enamel: - " the rhythmical deposition ("Fällung") of calcium salts in the colloidal medium of the organic ground substance, which process alters the organic matrix in some way in these (corresponding) layers" (WEIDENREICH,1926, p.344). FRISBIE, NUCKOLLS and SAUNDERS (1944) held that - " the markings of the enamel dependent upon the pattern of calcification are totally removed " by complete decalcification.

CZERMAK (1850) noted that the cross striae were more easily visible after treatment with dilute acid, an observation since confirmed by countless authors. von EBNER (1890) and PREISWERK (1903) adopted the unusual opinion that they were caused by the action of the acid.

ASPER (1916) noted that they were more easily visible (as also the Striae of RETZIUS) in sections which had been dried thoroughly before mounting in balsam.

BAUD and HELD (1956) reported finding the cross-striae in micro-radiographic images of normal enamel - most authors have regarded this finding as evidence of abnormality.

G.GUSTAFSON and PAYEN (1957 A,B) thought that polarised light microscopy - " allows of showing precisely the inequalities in distribution of the crystallites which correspond with variations in the mineralisation of the enamel". They concluded that there are organic septae crossing the prisms (accounting for the appearance of the cross-

8.11. striations) which are continuous with the organic prism-sheaths. (I can neither agree with their conclusions nor that they are valid deductions from the evidence they had available). A-G.GUSTAFSON adopts the same concepts - (1959), GUSTAFSON and GUSTAFSON (1960,1961).

DARLING (1958 p.133) argued from the nature of the changes during early caries of human enamel that it is "necessary to presume a segmentation of the cortex (outer wall containing insoluble organic material) to account for the cross striations".

Most workers have accepted that the cross-striations of the individual prisms add up to form straight lines, so that they correspond in adjacent prisms. (HERTZ, 1866; WENZEL, 1868. WILLIAMS, 1896A p.284, wrote that "the varicosities ..... are nearly always to be found arranged in regular rows"). But LEHNER and PLENK (1936) held that it was evidence of the individuality of the ameloblasts that the cross-striations " do not always lie at the same height" (they cited von EBNER, 1890 and MEYER ,1925). LEHNER and PLENK (1936,p.540) also summarised the present day majority opinion of the nature of the cross-striations ; they wrote that - "the cross striations and "Kittsubstanz" (prism-sheaths plus interprismatic substance) constitute a system of more weakly calcified, more highly organic regions of the enamel".

The nature of the rhythmic change of acitivity of the ameloblasts that is responsible for the production of the cross-striation of the enamel prisms remains unknown, but ASPER (1916) concluded that it was a "tolerable certainty" and a "great possibility" that "one cross-stripe and one resting - stage are formed in 24 hours, perhaps in direct relationship to sleep and awakeness". His opinion has been accepted fairly generally (GYSI, 1931; MEYER, 1935; SCHOUR and MASSLER , 1940, 1946; KOMAI, 1942; ORBAN,1957; and BOYDE, 1963). SCHOUR and MASSLER (1946) found that the cross-striation repeat distance, i.e. the daily increment, varies between 2 and 8μ depending on the site and tooth. KOMAI (1942) found that the cross-striation repeat distance was larger in deciduous than in permanent human enamel; and was reduced in the surface layers (confirmed by A-G. GUSTAFSON, 1959). He found that cross-striation was poorly marked in the enamel inside the neo-natal line.

Figure 8.1 .

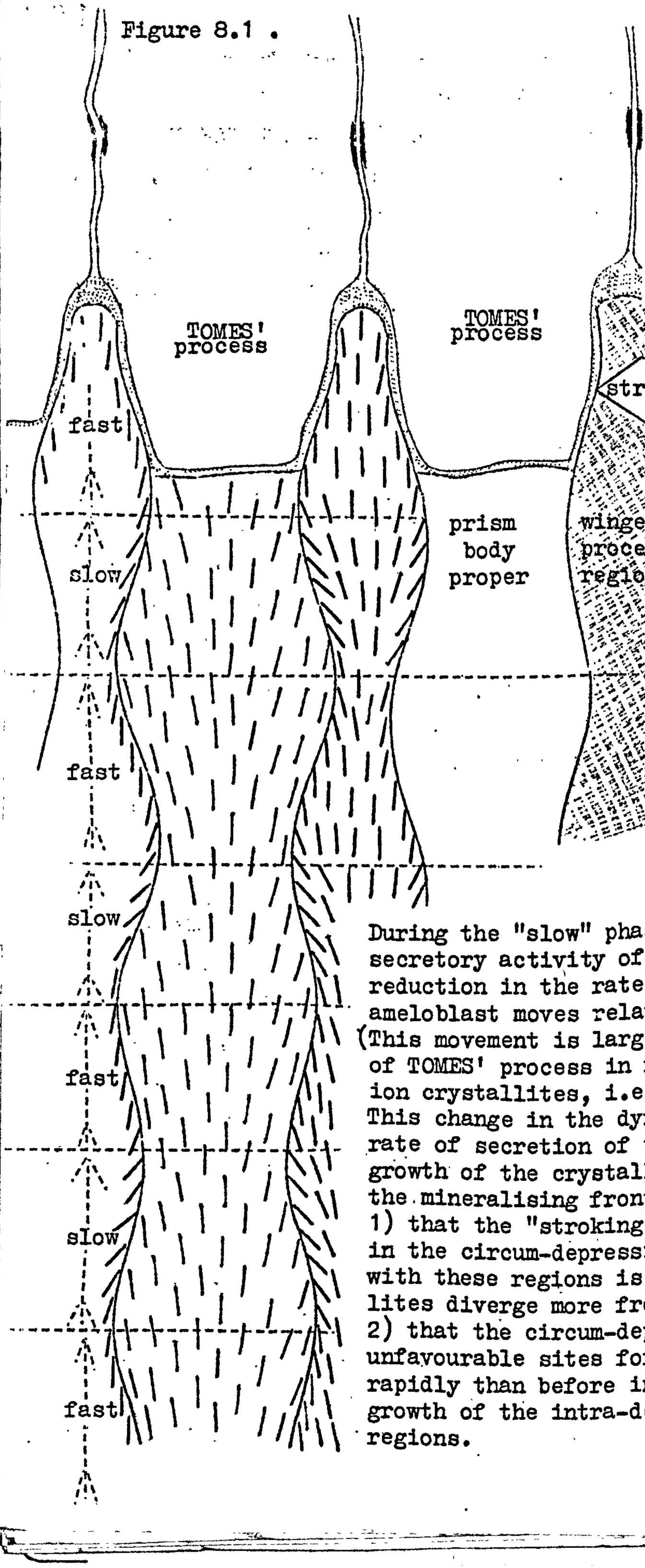


Diagram illustrating an hypothesis re the development of the cross-striations of the enamel prisms.

The diagram is based on the "Battlements" plane of section of Pattern 3 prisms (for Pattern 2 substitute "interrow sheet region" for winged process region).

stroking crystallite orientation factor operates here

the surface of TOMES' process moves past the stationary (circumdepression surfaces of the) mineralising front: the crystallites tend to be oriented in the direction of the resultant of this relative movement: the movement determines that this is a less favourable region for secretion and/or growth.

During the "slow" phases of the circadian cycles of secretory activity of the ameloblasts there is a reduction in the rate at which the surface of the ameloblast moves relative to the mineralising front. (This movement is largely confined to those surfaces of TOMES' process in relation to the circum-depression crystallites, i.e. the winged process regions). This change in the dynamic equilibrium between the rate of secretion of the ameloblasts and the rate of growth of the crystallites in the various parts of the mineralising front means: -

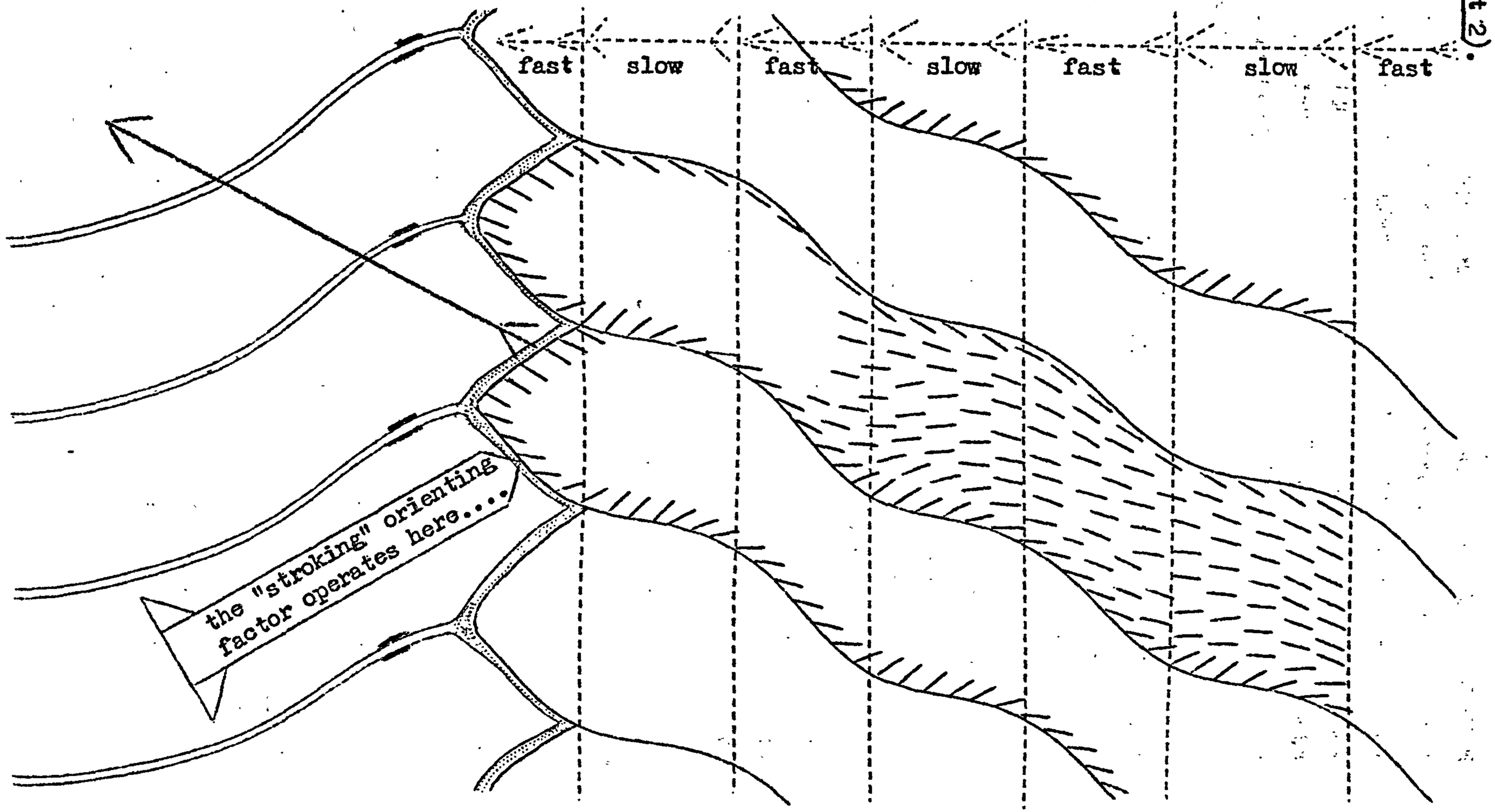
1) that the "stroking factor" orienting crystallites in the circum-depression regions to be more parallel with these regions is reduced, so that these crystallites diverge more from the "prism direction":and

2) that the circum-depression regions become less unfavourable sites for growth. Hence they grow more rapidly than before in comparison to the rate of growth of the intra-depression (prism body proper) regions.

8.12. HALBERG, HALBERG,BARNUM and BITTNER (1959) (and many others whom they quote) have demonstrated the wide occurrence of circadian (about 24 hours) rhythms in mammalian cellular systems. Most of the rhythms have been shown to be controlled by a rhythm inherent in the adrenal gland itself. The latter rhythm has a natural period of about 24 hours which is "tuned in" by the Photoperiod - or in the case of human beings by the complex of their daily cycle of activity. The only unusual feature of the daily rhythm of the ameloblasts is, then, that evidence of it is "fossilised" in their secretory product, the enamel - (this could be used to determine the rate of growth of fossil mammalian enamels).

The present view of the development of the cross-striations of the prisms finds considerable support from the work of HELMCKE,SCHULZ and SCOTT (1961,1963), which showed that the cross striations of the human enamel prisms are associated with periodic changes in the width of the prisms and the deviation of the crystallite c-axes from the axis of the prism. (They also point out that there is no electron-microscopic evidence published yet to show that there is any quantitative variation in the amount of organic material in the enamel matrix,which might correspond with the cross-striations). The crystallite orientation pattern in developing enamel is related to the rate of secretion of the enamel and to the shape (and orientation of) the mineralising front ; which is in turn determined by some sort of dynamic equilibrium between the secretion pressure (rate of secretion) of the ameloblasts and the growth of the "enamel". A rhythmic change of activity of the ameloblasts would cause a rhythmic change of shape in the mineralising front which might result in changes in: 1) the proportion of circum--depression crystallites (i.e. interprismatic in PATTERN I ; in the interrow sheets in PATTERN 2; and in the winged-process regions of PATTERN 3) to intra-depression crystallites: and 2) the deviation of the crystallites. (The change in shape of the mineralising front would determine a change in crystallite orientation. A change in the rate of secretion of the enamel would also result in a change in the proportion of intra-depression to circumdepression crystallites (see 8.1.) because of a reduction in the significance of the stroking

Figure 8.1. Diagram illustrating an hypothesis regarding the nature and mechanism of formation of the cross-striations of the enamel prisms. This diagram is based on the "Picket fence" plane of section of developing Pattern 2 prisms. During the "fast" phases of the circadian rhythm of secretory activity of the ameloblasts the more rapid movement of the surface of TOMES' process past the (stationary, semi-rigid) circumdepression surfaces of the depressions in the mineralising front determines that: 1) these become comparatively less favourable sites for crystallite growth and/or for secretion from the ameloblasts, and 2) the crystallites in these regions come to be oriented more nearly in the long axis of the prism.



8.13. orientation factor which would also allow a greater deviation of the circumdepression crystallites from the"prism direction"). These changes would produce a structure for the cross-striations (diagram figure 8.1.) very similar to that determined by HELMCKE,SCHULZ and SCOTT (1961,1963). It would differ in that these authors do not recognise an interprismatic substance, so that it is difficult for them to account for the "varicosities" of the prisms - i.e. the changes in the proportion of prism to interprismatic "substance". In order to accommodate the varicosities in their "model" these authors have had to assume a staggered arrangement for the cross-striations even as they are seen in transverse sections of the tooth: this does not fit the light microscope "facts".

Prism Segments and Boxes; non-existence thereof:- The present concept of the development of the cross-striations of the enamel prisms is at considerable variance with the opinions of A-G.GUSTAFSON (1959), SCOTT and NYLEN (1960) and NYLEN and SCOTT (1960). These authors believe that the cross-striations develop in segments of the ameloblasts which are separated off from the body of the cell at regular intervals. If such a process were to exist then we should expect to be able to find isolated segments of TOMES' process, completely separated from the surface of the developing enamel, in every plane of section which involved both ameloblasts and enamel. It has already been shown (Chap.2.4.) that this is not the case, since when the prisms are sectioned longitudinally - and in two mutually perpendicular planes - there are no holes to be seen in the enamel which are not continuous with the surface. A-G.GUSTAFSON's concept was useful to her because it explained the structure of the cross-striations as it has been envisaged by the GUSTAFSONS. The appearance for which she so aptly uses the term "Boxes", is the one which results when a section cuts the prisms obliquely, and portions of the depressions in the developing enamel front may be apparently separated from the rest of the surface. This appearance is entirely the result of the plane of section. A-G. GUSTAFSON (1959, p.26 and Fig.7) believed that her "boxes" could not "be artifacts caused by oblique sectioning of prisms (because).Prisms are hexagonal or round so that, however they are sectioned, their

14. corners cannot be right angles". The oblong impression of the projections of the developing surface seen in the "battlements" plane of section is not related to the shape of the prisms. It depends only on the parallelism of the projections of "interprismatic substance" (i.e. interrow sheet or winged process regions) and the flatness of the cervical side of the depression, i.e. the side from which the most "filling-in" occurs.

SCOTT and NYLEN (1960) based their opinions (Chap.10) on electron micrographs of the surface of developing murine inner-enamel. What they consider (by implication) to be longitudinal sections of the ameloblasts (e.g. Fig.22, p.418 of NYLEN and SCOTT, 1960; which is similar to my figure 2.17.) in fact cut the prisms almost transversely.

8.3. Surface Zone Enamel. The surface-zone enamel has received scant attention in comparison with its underlying bulk. Dental pathologists have been quick to realise that the surface layer of enamel of human teeth must, in some way, determine, allow, delay or alter the onset of caries of the enamel. It is a very difficult region to study because of the edge-artefacts associated with nearly every physical method which might be employed in the examination of sections cut at an angle to the true surface.

TOMES (1856,p.219) observed a "diminution of the fibrous character of the enamel at the termination of the fibres on the surface of the tooth, and also at the terminal edge of the enamel at the neck of the tooth". Studies of the early carious lesion in human enamel have amply confirmed the existence of this layer, and demonstrated its remarkable resistance to destruction during the early stages of decay (THEWLIS, 1940; GUZMAN, MERMAGEN and BRUDEVOLD, 1955; GUSTAFSON, 1957; DARLING, 1958). LOE and RAVNIK (1961) have demonstrated this layer in dog enamel; TOMES (1850) showed it in rodent enamel; and my own observations suggest that we may suppose it to be of fairly general occurrence in mammalian enamels.

A number of workers using micro-hardness testing methods, have found the surface-zone enamel to be harder than the subsurface enamel (HODGE and McKAY, 1933; HINDS, 1943; NEUBRUN and PIGMAN, 1960).

3.15. JEFFERY (1932) found the surface layer (ca. 5μ)of dog enamel to be the most impervious to dyes.

HÖLLANDER and SAPER (1935) noted an apparently more x-ray absorbent surface layer, at the edge (i.e. true surface of the tooth) of their microradiographs of enamel. They concluded that it was a photographic artefact produced during the development of the (silver) image - a "Mackie" line. THEWLIS (1940), APPLEBAUM (1940) and BAUD and HELD (1956) concluded that this line was occasioned, at least in part, by a real increase in the mineral content of the surface zone enamel. AMPRINO and CAMANNI (1956) found high x-ray absorption in a thin (50 - 100 μ) superficial layer in human and dog enamel. HODGE, VAN HUYSEN and WARREN (1937),THEWLIS (1940), SONI and BRUDEVOLD (1959), BRUDEVOLD, STEADMAN and SMITH (1960) and ANGMAR, CARLSTRÖM and GLAS (1963) have demonstrated a steady, gradual decrease in the degree of mineralisation of the enamel from the surface zone towards the enamel--dentine junction. ANGMAR, CARLSTRÖM and GLAS (1963) believe that the hypermineralised surface zone is a photographic-development artefact.

GLAS and OMNELL (1960) studied the surface enamel of a Hippopotamus lower canine by x-ray diffraction. They found that - "The apatite crystallites in the outer part (200 μ) of the enamel were extremely well-aligned so that their c-axes run roughly perpendicular to the large flat areas of the natural surface of the enamel".

The"ion etching" studies reported in Section 5.8. (and in BOYDE and STEWART, 1962A,1962B and STEWART and BOYDE , 1962) showed a uniform rate of erosion in the surface zone enamel in Macropus. This result is consistent with a higher degree of preferred orientation of the crystalline component and uniformity in the degree of mineralisation in the true surface zone.

The present electron-microscope studies (Chapter 2) showed a preferred orientation perpendicular to the "mineralising front" for the crystallites in the developing surface zone enamel.

Direct examination of the surface of human teeth reveals the existence of the "circular wrinkles" (LEEUWENHOEK,1678) and the "enamel rod ends" (EISENBERG, 1938; HELMCKE and RIENITZ, 1959). WOLF

B.16. (1940) and SCOTT and WYCKOFF (1946) have shown that there is a general correspondence between bands in which the rod ends show and do not show, and the troughs and crests of the Perikymata: the enamel "rod ends" show in the troughs of the Perikymata. The appearance of the rod ends on the surface of the enamel is rather more random in Canis familiaris, but these teeth are without Perikymata (WOLF, 1942 B). The correspondence between the Brown Striae of RETZIUS and the troughs of the Perikymata was demonstrated by PANTKE (1957).

I would like to propose the following correlation of the above observations. During normal, undisturbed enamel development, all those ameloblasts which have nearly completed the secretion of the full thickness of the enamel for which they are responsible, simultaneously slow down or in some way undergo a diminution of their secretory activity; "lose" their TOMES' processes; and finally stop enamel formation altogether. The evidence that amelogenesis slows down to a stop, rather than halts abruptly, may be seen in the gradual reduction in the cross-striation repeat distance in the sub-surface enamel (KOMAI,1942; A-G.GUSTAFSON,1959, p.81., wrote - "A relatively broad layer under the surface often consists almost entirely of compressed sections"). This gradual cessation of secretory activity is associated with the development of a surface layer (domain)of enamel in which the crystallites are all parallel and oriented at right angles to the surface, and in which there are, therefore, no "prisms"; there are also the absolute minimum of signs of the impressions of the inner-ends of the ameloblasts - (for this is the true nature of the so-called "enamel rod ends" - it was shown elsewhere (2.4.) that the correspondence between one depression in the enamel surface and one prism is by no means of universal occurrence). During the periods in which some imperfectly understood factors determine the development of the incremental lines of RETZIUS, the ameloblasts which were in the phase of a gradual cessation of secretory activity suffer a sudden early and complete stop to their secretory activity. This sudden stop in enamel formation may be seen as a circular deficiency on the enamel surface, i.e. as a trough. It is associated with the absence of the surface - layer (parallel crystallites : prism free) enamel and the

8.17. presence of marked impressions of the inner ends of the ameloblasts ("enamel rod-end impressions"). In those mammals in which the incremental lines are almost parallel with the enamel surface (i.e. in which a large surface area of enamel develops at the same time in every tooth), e.g. in the dog (WOLF, 1942 B), the degree of prominence at any particular site of the "enamel rod-ends", or vice versa, the surface zone enamel, depends on whether the ameloblasts underwent an "abnormal" accelerated, or a normal gradual cessation of enamel formative activity.

The results obtained from the study of ultrathin sections of the surface-zone enamel; from the argon-ion etching study; and the published results obtained by x-ray diffraction and polarised light microscopy (see Chapter 1) would seem to suggest rather strongly that the "prismatic" structure is absent in the true-surface zone of most mammalian enamels. Put in another way, the crystals are all approximately parallel and there are therefore no planes of junction between domains containing crystallites in different orientations (i.e., no prism boundaries). This structure might be regarded as an accidental by-product of the mode of formation of enamel or as having a definite functional significance. The parallel array of the crystallites might possibly allow a more perfect mineralisation of this zone, and this might further be assumed to be the case on the grounds of the proximity of this zone to the source of "minerals" during maturation. In addition, the absence of "organic prism-sheaths" from the surface zone supports the concept of an intact surface "seal" on a completed tooth. This natural, highly inorganic seal sits below the organic seal - the enamel cuticle. There are reasons for believing that (in the human at least) both types of seal are lost as a result of functional attrition, but that both are replaced to some extent by other formations. The enamel cuticle is replaced by a derivative of salivary debris, and the surface zone enamel by an amorphous BEILBY layer formed during the actual attrition.

8.4. Reptilian Enamel. POOLE (1956) made a very interesting proposal about the development of mammal-like reptilian enamel in which a pseudo-

3.18.

-prism structure may apparently occur. In writing of the structure of reptilian enamel, he stated -"Other synapsid teeth possessed a thin, well-defined enamel layer made up of incremental lamellae but lacking true prisms. Nevertheless, cylindrical groups of crystallites exist throughout this enamel within which the orientation of the crystallite axes varies regularly. Between the crossed nicols of a polarising microscope this crystallite arrangement gives the enamel a prismatic appearance. However, the enamel is quite homogeneous, for these pseudo-prisms are not physically separated from each other. Furthermore, this regular prismatic appearance and an irregular Säulenliederung such as exists in crocodile enamel, may exist in the same tooth. There is, therefore, no evidence that the prismatic enamel characteristic of mammals existed in the pre-mammalian reptiles". POOLE suggested that the crystallites develop at right angles to the surface of the developing enamel and that a regular, shallow, three-dimensional corrugation (i.e. shallow depressions) of the surface existed; so that although there are changes in crystallite orientation there are no abrupt changes, and there is no clear delineation between domains in which the crystallite orientation changes. He wrote of a - "continuous homogeneous layer of crystallites, the orientation of which varies slightly but regularly". POOLE's ideas are very interesting, because they would place mammal-like reptilian enamel in an intermediate position between reptilian and mammalian enamels as regards the degree of development of the depressions in the developing enamel surface which are regarded as responsible for the development of prisms in mammalian enamel in the present thesis.

POOLE (personal communication, 1963) has suggested that the whole thickness of crocodilian enamel, which is prism-less (ERLER, 1935; SCHMIDT and KEIL, 1958) is equivalent in most respects to the surface zone of mammalian enamel. This exceptionally interesting concept is supported by my own limited findings on Caiman enamel (figure 2.30.) which would suggest that there are no TOMES' processes (finding supported by KVAM, 1946) and that the crystallites all develop approximately perpendicular to the mineralising front.

8.19 8.5. The orange pigment in rodent incisor enamel was commented on by CUVIER (1825), RETZIUS (1937), ERDL (1841), OWEN (1845) and many others. von BIBRA (1844) concluded that the yellow colour was due to the presence of iron oxide - a result which was cited by many later workers. WENZEL (1868) described the histology of the deposition of the yellow pigment granules into the enamel for the first time. The iron pigment has been studied in normal and pathological conditions by McCOLLUM,SIMMONDS,BECKER and BUNTING (1925) ; WOLBACH and HOWE (1925, 1933); KORVENKONTIO (1934-35); MOORE (1943); MOORE and MITCHELL (1955); PINDBORG,PINDBORG and PLUM (1946); PINDBORG (1950,1953); BUTCHER (1953); and DAM and GRANADOS (1945)- see BOYDE (1964 MS) for details.

Our own determinations of the iron content in the true surface layer of rat incisor enamel (BOYDE, SWITSUR and FEARNHEAD, 1961) are the first reported values from analyses accurately confined to this layer. Our finding (BOYDE and SWITSUR -unpublished) of iron in the orange-pigmented surface layer of shrew molar enamel, and coypu (Hystricomorpha) and squirrel (Sciuriomorpha) incisor enamel make it seem certain that all orange pigmented enamel will be found to contain a substantial proportion of iron. The high content of iron (7.6%), and the colour which it imparts (i.e. orange, as against green) would suggest that the metal is present as the ferric ion.

8.6. Enamel-Dentine Junction. Many workers have accepted the existence of a layer of poorly calcified "enamel" at the enamel-dentine junction,continuous with the interprismatic substance (e.g. ORBAN,1925, A; GUSTAFSON, 1945). It is not certain whether this is identical with the enamel-dentine membrane of some older workers (BERZELIUS, ca.1802 cited by ANDRESEN, 1902),i.e. a membrane which can be raised (isolated) in the vicinity of the enamel-dentine junction after the violent acid decalcification of the tooth. The dentino-enamel membrane seems to be an invention to explain the adhesion of enamel and dentine. It has been supposed that the ameloblasts are responsible for secreting this dentino-enamel membrane (membrana limitans) before they commence their major activity of producing the enamel. The differently staining layer which has been so identified is probably equivalent to the first formed extra-cellular granular component (pre enamel - not mineralised) of the

8.20. enamel "matrix". Modern adhesion theory suggests that two different materials will adhere if there is an intimate "molecular" contact between their opposing surfaces. It is therefore unnecessary to assume the presence of an adhesive layer, since enamel and dentine develop in contact with each other.

Enamel does not cleave off the surface of the dentine when a freshly extracted tooth is fractured, but it does so with greater ease if the tooth is allowed to dry - this is particularly the case when a tooth specimen is force-dried, by heating or under a reduced pressure. This failure of adhesion of the two tissues is the result of the difference in the amount of shrinkage which occurs when they dry and this is probably the result of the much higher free water content of the dentine. It is more likely that the cleavage will occur within the more brittle tissue, i.e. in the enamel. This is observed in practice - see figure 4 p.191 of BOYDE, SWITSUR and STEWART (1963) and figure 5 p. 167 of BOYDE and STEWART (1962A). The plane of cleavage between the two tissues cannot be assumed to be exactly at their plane of junction, and this cannot be regarded as a reliable method of separating the two tissues, prior to biochemical studies for example.

8.7. Translucent Zone in Enamel at Enamel-Dentine Junction. A thin translucent zone in the enamel just inside the enamel-dentine junction has been accepted as a more perfectly (highly) mineralised layer by many workers. GUSTAFSON (1945) suggested that its higher (negative) birefringence (first noted by von EBNER, 1906) was due to the prisms in this area all travelling in the same direction. FANIBUNDA (1957) suggested that the appearance of the translucent zone could be partly accounted for by the reflection of light from the enamel-dentine junction, since he found its width to be dependent on the angle of incidence of the (transmitted) light. This "zone" is the "first formed layer of enamel", and its translucency would be explained by the high degree of preferred orientation of the crystallites (section 2.4.4., figure 2.29.) and by its possibly more perfect mineralisation; both of which would lead to a reduction in the size or proportion of (light scattering) spaces containing material of a lower refractive

8.21. index in this region. ANGMAR, CARLSTRÖM and GLAS (1963) were unable to find the "highly mineralised zone adjacent to the dentine enamel junction, which has been presumed to exist because of the strong negative birefringence usually seen in this area".

A slightly higher mineral content (Ca K_α emission) was found in this region in the rat incisor (BOYDE, SWITSUR and FEARNHEAD,1961 p. 204, Fig.2b). The enamel-dentine junction domain has proved difficult to study with the ion-etching technique because the tissues have cleaved apart (in the enamel) near the junction on drying in vacuum (Fig. 4, p.191 in BOYDE, SWITSUR and STEWART, 1963; Fig.4, p.167 in BOYDE and STEWART, 1962 A . and Fig. 5.8 of this thesis).

8.8. SUMMARY and CONCLUSIONS (CHAPTER V111)

1) Incremental lines (the "brown"striae of RETZIUS) and the troughs of the Perikymata in which the true surface zone (domain) enamel does not develop are related to a systemic influence which reduces the rate at which "enamel" is secreted. The colour of the "brown" striae must be related to their containing larger (blue light scattering)"spaces" than the surrounding enamel, and probably fewer - though of large diameter - crystallites per unit volume.

2) The details of development of the cross-striations of the enamel prisms have never been revealed. Some of the experimental work reported in this thesis shows that the appearances held as revealing the development of these structures by, for example, ABBOTT (1889), GUSTAFSON (1959) and NYLEN and SCOTT (1960), have been misinterpreted. These workers have regarded rows of transversely sectioned prisms as rows of cross-striations.

It is postulated that the cross striations are associated with periodic variations in crystallite orientation along the length of the enamel prisms (which can include a variation in the proportion of "interprismatic substance") which are associated with variations in the shape of the mineralising front and/or the rate of secretion of enamel precursor substances. This conclusion is not in conflict with any of the known facts about the cross-striations. Their increased visibility after partial acid decalcification is probably due to selective

8.22 dissolution of differently orientated crystallites, but an associated change in the composition or amount of the organic matrix cannot be rigidly excluded.

3) The formation of the true surface (zone) domain occurs during the gradual reduction in rate of secretion of enamel associated with the disappearance of TOMES' processes. It is absent when a group of ameloblasts cease enamel formative activity prematurely and abruptly. The depressions in the surface of the developing enamel occupied by the TOMES' processes are then retained in (a trough in) the surface of the enamel.

TABLE 9.1

Summary of previous views on the position and origin of the "tubules" in marsupial enamel.

(For further details see BOYDE, 1964MS).

		+ = dentinal origin	+ = continuous with dent.tubules	+ = enamel origin	+ = situated in prisms	+ = situated in interprism.subst.	Name adopted (CANAL = Kanalchen, canal, or canaliculus).	+ = developing tissue studied	Other remarks
A	J.TOMES (1849,1856)		+	+			TUBE& FIBRIL		note A
B	von EBNER (1890)		+	+	-	+	CANAL.		note B 3-5 round each prism
C	C.S.TOMES (1897,1904)		+	+	+	-	FIBRE& FIBRIL	+	note C 1 in every 4 prisms.
	"ROSE (1893, 1897)	+	+		-	+	CANAL.		
	PAUL (1896)					+	TUBULE		
	WILLIAMS (1897,1923A and cit.by CARTER,1920)	+	+		+ 1/4	+ 3/4	FIBRIL, CANAL		
D	WALKHOFF (1898)				note D		CANAL		denied existence of "Kittsubstanz"
	MUMMERY (1914,1915,1919)	+	+			+	TUBE & FIBRIL	+	penetration of dentinal fibril
	ADLOFF (1914)					+	CANAL		
	CARTER (1918,1920,1922)				+ 1/4	+ 3/4	TUBE & FIBRIL	+	direction not dependent on prisms
	WEIDENREICH (1926)	+	+		+ 1/2	+ 1/2	CANAL		
	MUNCH (1929)						CANAL		
	SPRAWSON (1930)		+			+	TUBE		stain penetration d.ts. - e.ts.
	MARCUS (1931)	+	+		+		axial FIBRIL		
	"HÄUSELE (1932)				+				
	SKUES (1932)			+	+		FIBRIL	+	
	McCREA and ROBINSON(1935-36)		+						stain penetration d.ts. - e.ts.
E	MOSS and APPLEBAUM (1963)		-	+	+		FIBRE	+	= to tufts, (noteE) uncalcifed rods.
	BOYDE (1964)		+	+	+	?	TUBULE	+	deficient crystal formation locally

A) "Indeed in all teeth the enamel fibre is at an early stage of formation partially tubular". (TOMES, 1849, p.403)

B) Von EBNER invoked a resorption of the first formed dentine to account for the continuity with the dentinal tubules. He also described the positive birefringence of marsupial enamel for the first time.

C) Noted that the "honeycomb" of developing enamel is of greater thickness in marsupials.

D) Canals not bound to individual prisms.

E) "Tubules" are ground section artefacts.

9.1.

C H A P T E R IX

ENAMEL TUBULES ; SPINDLES ; TUFTS and LAMELLAE. (DISCUSSION).

9.1. Marsupial enamel "tubules" or "fibres".
9.2. Enamel spindles.
9.3. Tufts and lamellae.

9.1. Marsupial enamel tubules. A detailed account of the work of previous authors relating to the structure and/or development of marsupial tubular enamel is given by BOYDE (1964 MS): a summary of their main findings or opinions is given in the accompanying Table (Table 9.1.).

Results reported in this thesis show:—

1) that at least some of the so-called "enamel tubules" are continuous with the dentine tubules (5.10.1).

2) that the"tubules" leave more residue (i.e. the"fibre" of TOMES, 1856) than the surrounding enamel when decalcified (2.4.3.7.)

3) that the "tubules" are permeable to dyes in extracted teeth (5.10.1).

4) the dyes methyl blue and trypan blue did not reach the enamel "tubules" from the pulp or blood-stream in in situ adult (Didelphis) teeth (5.10.2.).

5) the "tubular" nature of the"tubules" is well demonstrated in the replicas* taken from the argon ion-beam eroded Macropus molar enamel surface (Section 5.8 and figure 5.7).

6) the tubules are situated mainly within the "prisms".

7) the tubules may be recognised in electron micrographs of

*Professor J.G.HELMCKE (Forschungsgruppe für Mikromorphologie der Max Planck Gesellschaft, W.Berlin) was kind enough to allow me to examine his electron-micrographs of replicas of fractured marsupial enamel, and from these it would appear that the tubules have clearly defined walls.

NOTE

Enamel "tubules" (rather than the spindle-formed dilatations that are found in human enamel, for example) are also found in certain members of the Orders Rodentia (e.g the Jerboa - TOMES, 1849; Von EBNER, 1890), Insectivora (TOMES, 1849 -.e.g. hedgehog, mole, shrew), Primates (CARTER, 1922 - N.B. the Lemuroidea), and Cheiroptera (LOHER, 1929, Beitrag zum gröberen und feineren (submikroskopischen) Bau des Zahnschmelzes und der Dentinfortsätze von Myotis myotis, Z. Zellforsch. 12, No. 4). The marsupials are a special group in respect of their possessing "enamel tubules" in that only the one exception noted by TOMES (1849) has ever been reported (i.e. the wombat does not have enamel tubules).

I have examined the ground sections of cheiropteran teeth in the TOMES' collection of the Royal College of Surgeons of England (in the Odontological Museum - I am indebted to the Curator, Professor A.E.W. Miles for permission to study this material) and can confirm that tubules are present in some species, e.g. Pteropus poliocephalus, Magaderma lyra, Barbastellus communis, and more questionably in some others.

9.2. developing enamel as regions in which crystallites do not develop (2.4.3.7.).

8) The study of the development of these structures (section 2.4.3.7.) revealed no special features of the ameloblasts or of the nature of the first secreted enamel - other than a confirmation of the observation of TOMES (1904) that the "honeycomb" -"in enamels which are to have solid prisms - occupies only a very small vertical depth, while in tubular enamels it is of material thickness".

The present results, therefore, lend support to both the "tubular" and"fibrillar" concepts of the structure of the "tubules": the name "tubule" will be adopted on the rather arbitary basis that it is more suggestive of their nature as defects in a more or less continuous phase of "enamel". The tubules are "defects" in the sense that they contain fewer (or no) crystallites than the surrounding enamel.(Use of the term defect is not meant to exclude the possibility that a factor causing the absence of the mineral component may reside in the organic matrix in the first place and is not meant to imply that the genesis of the tubules is necessarily accidental). To state that the tubules are regions of the enamel which do not "mineralise" fully is only to summarise their nature : this does not begin to provide an explanation of the mechanism of their development or of their adult function. Although the present study can provide no final answer to the question of why the tubules develop in marsupial enamels, their various and characteristic features suggest a number of alternatives, which will be considered next in turn. Structures related to the enamel tubules - both actual tubules in the Muridae, Sciuridae, (von EBNER, 1890); and more amorphous spaces in the Hystricomorpha (TOMES , 1850; KORVENKONTIO, 1934-35) have been described in rodent enamels: these are not continuous with either the enamel-dentine junction or the enamel surface. The fact that these rather similar regions in rodent enamels (i.e. the "cells" of TOMES, 1850) and the tubules in some marsupial enamels (e.g. in the cervical region of Petaurus molar enamel - TOMES, 1849; von EBNER, 1890) are not continuous with the dentine tubules, would suggest that the

9.3. <u>predisposing</u> cause of the development of these "defects" does not lie in connection with the surface of the dentine. In the development of those enamels in which the dentine and enamel tubules are continuous, we must simply consider that the predisposing cause is present at the earliest stage of formation of any given increment of enamel, i.e. when the first enamel crystallites develop on the surface of the dentinal collagen matrix. However, it is quite probable that some extra <u>initiating</u> factor in enamel tubule development resides at the future enamel - dentine junction in marsupials, and that this combined with a predisposition to the development of "defects" (in crystallite formation) determines the very high frequency of the tubules "crossing" the enamel-dentine junction in the teeth of marsupials.

The fact that a high proportion of the enamel tubules appear to be in direct continuity with the dentinal tubules certainly might suggest that their presence may depend in some way on an "influence" from the dentinal tubules (the odontoblast processes). It must then be determined why the odontoblast processes persist so near to the enamel (in the groups that possess tubular enamel with continuous enamel and dentine "tubules") and what the postulated "influence" is. There is no doubt that the so-called "enamel tubules" are purely enamel formations - there is no evidence for their containing a morphologically identifiable dentinal component, as, for example, collagen fibres. However, it is possible that the growth of the first enamel crystals at the enamel-dentine junction depends, in some way, upon the presence of an underlying substrate of dentine: calcified or not. The formation of "enamel tubules" might then be initiated by the presence of defects in the outermost layer of dentine, such as we must presume to exist if the odontoblast processes actually reach to and remain in contact with an imaginary amelo-dentinal contact plane.

It is conceivable that the initial nucleating factor which starts <u>enamel</u> crystallite formation at the enamel-dentine junction resides in the collagen fibres of the dentine matrix. Since these collagen fibres lie predominantly perpendicular to the surface of the dentine, and apatite crystallites are known to form on (or in) the surface of collagen fibres (references - FRANK and NALBANDIAN, 1963), an epitactic mechanism here might explain the degree of preferred orientation observed even

9.4. in the first formed layer of enamel. Deficiencies in the surface of the dentine would result in "nucleation deficiencies" for the enamel crystallites. Defective territories in which crystallites did not commence to grow (i.e. the "tubules") could only be propagated in regions in which the crystallites were parallel and essentially at right angles to the dentine surface, i.e. mainly within the prisms. However, the crystallites in Macropus interrow substance are essentially parallel for considerable distances and some defects might therefore continue to be formed in a direction at a large angle to the prisms, and not contained "within a prism". Such an explanation presupposes that the first formed enamel crystallites traverse large distances (as far as the tubules are long) through the enamel before they abut against a field containing crystallites having a different orientation (at a future "prism-sheath" plane), and that new crystallites are rarely formed. This is probably not the case.

The observation (2.4.3.7.) that the enamel"fibres" only appear as structures differentiated from the remainder of the decalcified enamel matrix in the "transitional" (CHASE, 1935) stage of maturation suggests that the "tubules" (fibres) acquire their high organic content as a result of "maturation". This would make their acquisiton of an increased organic content - more resistant to acid decalcification - analagous to the development of the "prism-sheaths": the organic matrix being forced into these regions during the growth in diameter of the enamel crystallites. The enamel "tubules" have a much greater diameter than the prism sheaths and could contain a greater bulk of organic material: this would explain the greater mechanical stability of the content of the tubules (i.e. the "fibre" of TOMES, 1856) after acid decalcification.

It seems very probable that the development of marsupial enamel tubules and rodent enamel "cells" (TOMES, 1850) is associated with the extremely rapid deposition of these tissues; and is, as it were, an accidental effect of this cause. The experimental data of SCHOUR and his collaborators (references in section 8.1) re the rate of

9.5. deposition of rodent incisor enamels (viz: 16 μ per day) is probably quite acceptable : and this rate contrasts strongly with that in man, i.e. an average of 4 μ per day (SCHOUR and MASSLER, 1946). CARTER (1917) and MUMMERY (1919) noted that Macropus enamel was still "soft" throughout after the shape of the crown had been attained - MUMMERY noted the enamel to be three times as thick as the dentine at one place in a Macropus tooth and that all of this enamel stained well with methylene blue.

CARTER (1917, p.294) stated that -"the enamel of Higher Mammals is usually laid down slowly, and coincidently undergoes almost complete calcification. In Marsupials, however, this is not the case, for the enamel matrix is laid down very rapidly, practically the whole thickness of the tissue being deposited, whilst but a slight amount of dentine has been formed". MOSS and APPLEBAUM (1963, p.293) also -"suggest that the apparently very rapid rate of enamel matrix formation may play a role in the production of this type of enamel". A surprising difference was found in the amount of enamel formed in the dentition of two sibling pouch young Didelphis nudicaudata , the one killed 7 days after the other (Appendix B). If the length of the TOMES' process is in any way an indication of the secretory activity of the ameloblast then the greater length of the marsupial TOMES' process (C.S.TOMES, 1897, 1904) might indicate a greater secretion pressure (in the sense of a greater rate of activity) of the marsupial ameloblasts.

MOSS and APPLEBAUM (1963) consider themselves to have shown that the enamel tubules (A term which they decry, without offering any suitable alternative) in Macropus are in fact uncalcified enamel rods; and that they are therefore equivalent to the tufts in other mammalian enamels. Whilst agreeing with these authors that the tubules are purely enamel formations, I cannot consider that they have made any real contribution by naming them as "rods". Their work is likely to add to the confusion on this subject, since their idea of the size of the tubules (i.e. approximately that of the prisms themselves) is quite out of touch with any previous descriptions. Further , they

Figure 9.1.

Electron micrograph (X 14300) of longitudinal section of pouch-young Didelphis nudicaudata (rat-tailed opossum) tooth germ, showing parts of two prisms (and a small part of a third prism in the bottom of the field) and two TOMES' processes in a "Picket fence" plane of section. CUSPAL direction = SE. The crystallites in the far cervical region (i.e. towards the top of the field) of the prism in the lower half of the field show a divergence of ca. 60° from the long axis of the prism: this divergence is greater than has been observed in other Pattern 2 enamels. The variation in the width of this prism (i.e. in the profile of the prism boundary plane running across the centre of the field) is of the nature considered in Figure 8.2 in connection with the current hypothesis regarding the development of the cross-striations of the prisms, and figured by HELMCKE, SCHULZ and SCOTT (1961, 1963).

MECKEL, A.H., GRIEBSTEIN, W.J. and NEAL, R.J. (1964 - in the Proceedings of the International Symposium on the Composition, Properties and Fundamental Structures of Tooth Enamel held very recently in London, in their paper on the "Ultrastructure of Fully Calcified Human Dental Enamel") have reported divergences of up to 70° in the cervical "tail" of Pattern 3 prisms. (Their name "tail" for this region is eminently suitable and is far more likely to "catch on" than the resurrection of the term "winged process" that I have suggested.)

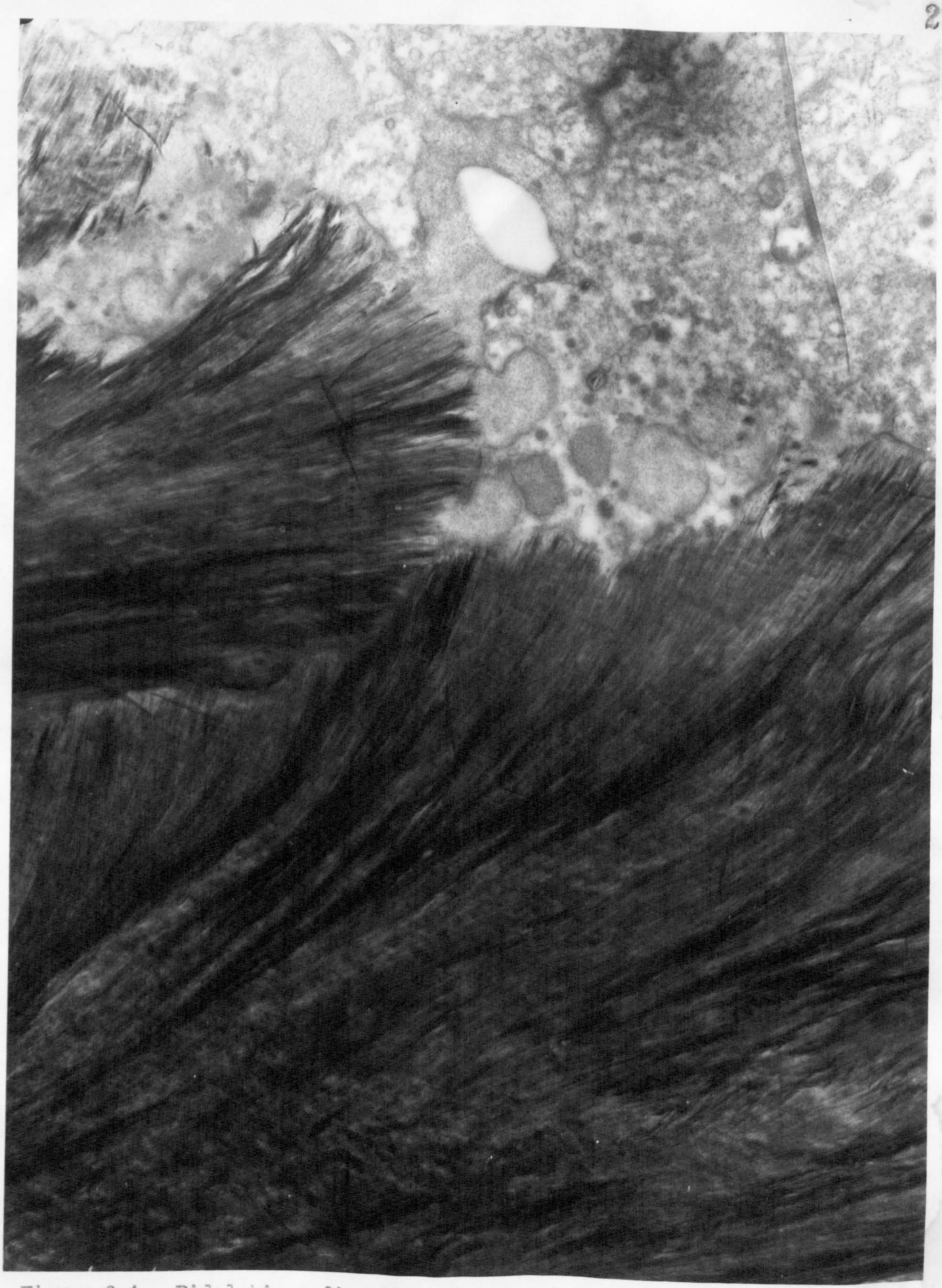


Figure 9.1. Didelphis nudicaudata: "Picket fence" L.S. prisms. (X 14300)

Fig
9.1.

9.6. fail to offer any explanation of why individual rods should calcify imperfectly; and one would get the impression from their illustrations (if one would believe that the outlines of prisms in a decalcified section are equivalent to the tubules) that the enamel must consist largely of "tubule" material and therefore be highly organic.

MOSS and APPLEBAUM do not believe that there is any continuity between the enamel and dentine "tubules". They state that (1963,p.294) -"considering the abundance of both dentinal tubules and of enamel fibers in the Marsupial, it is little wonder that the illusion of continuity between them is achieved with the use of relatively thick ground sections" and also (loc. cit) that -"the enamel"tubules" are an artifact of ground section preparations, and,further, that in vivo they contain uncalcified enamel matrix rather than the continuation of any odontoblastic process". In the sense in which I have used the term tubule, there is certainly no justification for considering them as artefacts: the conclusion that they contain uncalcified enamel matrix is in agreement with my own findings.

von EBNER (1890) found that Macropus enamel was positively birefringent - this was confirmed by SCHMIDT (1924). POOLE (1960)

9.7. reported that Macropus and Didelphis enamels were, in fact, weakly negatively birefringent in water, but became positively birefringent in parts when imbibed in alcohol. This difference in the birefringence of marsupial (tubular) and other mammalian enamels may well be associated with:-

1)a greater divergence of crystallite from prism axes in the former. The observed birefringence depends to a large extent on the degree of preference for a given orientation of the individual crystallites: this is reduced if the crystallites diverge to a greater extent about, and on either side from,the prism axes. [The present electron microscopic results tended to show a greater divergence of the crystallites in marsupial enamel (Figure 9.1.)] and

2)a comparative deficit in the total amount of mineral in the marsupial enamel, leading to a reduction in the negative intrinsic birefringence of the apatite component. This would be in keeping with the rapid growth - or delayed maturation ! - intimated above.

9.7

9.2. Spindles. The enamel spindles cannot be regarded as structures analogous to the enamel tubules, since there is good evidence that the spindles are, or have, dentinal components. (FRISBIE, 1952; SCHLACK, 1940). They are also much thicker ; being greater in diameter than the prisms - and much shorter ; reaching only some 50 - 100 μ from the dentine surface. The presence of spindles has been noted - or that of a similarly shaped structure in a similar situation - between the ameloblasts just before the commencement of amelogenesis (LAMS,1920; CHASE, 1948; and others); whereas the tubules develop as faults in the process of filling in of the prisms (i.e. as an absence of crystallite growth in limited territories within otherwise normal domains) during enamel development. (SCHMIDT (1963, B) holds that the spindles are formed by the resorptive expansion of dentinal tubules at the expense of already calcified enamel prisms.)

The material used in the present study has not contributed any information towards the solution of this particular problem. Nevertheless, it seems reasonable to speculate on the basis of what is already known, that the "spindle-material" (be it initially either dentinal matrix, i.e. connective tissue intercellular substance, or odontoblast cytoplasm) penetrates between the inner ends of the inner enamel epithelium cells, before their inner-end terminal bars are complete, that is, just while amelogenesis is getting under way

9.8. over the tips of cusps. The"spindle-material" would find a block to its progress in the form of the outer terminal bars of the ameloblasts, which are well developed and very prominent from a much earlier stage. If this hypothesis were correct, one might expect to find some correlation between the length and direction of the spindles and the length and direction of the ameloblasts adjacent to the dentine surface. The lengths of the spindles (ca. 70 μ) agrees well with that of formative ameloblasts, and their direction at right angles to the enamel-dentine junction (rather than parallel with the prisms, which they "cross" - A-G. GUSTAFSON, 1949) is again parallel to the known axes of the ameloblasts at this stage of formation. The additional presumption that the inner terminal bars are only "late" in forming in the cells overlying the tips of cusps (or incisal edges) during the beginning stages of amelogenesis in any given (human) tooth would explain their peculiar distribution. Unfortunately, the published work which deals with the question of the formation of the inner terminal bars (in human amelogenesis) is not very precise with regard to the details of their time of formation in relation to the commencement of dentine and enamel formation.

ESCHLER (1938) reported that the inner terminal bars in pig foetal ameloblasts only develop after dentine formation has commenced. A closer study is required; one which would attempt to correlate possible differences in the time of formation of the inner terminal bars in different species ; in different teeth; and in different regions of the same tooth, with the frequency of occurrence of spindles.

9.3. Tufts and Lamellae. The results of previous studies of the structure and/or development of the tufts and lamellae were given in Chapter I.

The results of the argon ion beam erosion study (5.6.) confirm the results of the previous workers (cited in Chap. I) that the tufts (and lamellae) are poorly calcified areas. The enamel in tuft-areas was eroded away more rapidly than the surrounding normal enamel: this could be due to a difference in crystallite orientation or

.9. composition, but the latter is more likely in this case. (This result fits the general conclusion (BOYDE and STEWART, 1962) that more poorly mineralised structures were eroded away more rapidly under the particular experimental conditions which were used).

Fig. 6a on p. 169 of BOYDE and STEWART (1962) shows a stereoscopic view of an etched tuft. It can be seen that the tuft is several prisms wide, and involves both "prism" and "interprismatic" regions: this dispels the impression that either one or other "substance" is particularly involved in the formation of tufts, and it also reveals that the tufts are not narrowly confined structures - this particular "tuft" has a width of some three or four prisms. Tufts and lamellae are thus regions of defective mineral content, and it must be decided whether this difference is due to a difference in crystallite size or intercrystalline spacing.

The present electron-microscopic results suggest that it is unlikely that new crystallites grow other than at the developing, mineralising front in enamel. A"normal" mineral content in enamel is thus achieved by the crystallites growing in diameter - this might be prevented by:

1) a "poisoning" of the growing crystal surfaces

2) a limited availability of ions for further crystal growth

3) the abnormal persistence of the organic component as a result of a change in its nature, or a mechanical blocking of its removal.

or 4) a change in the organic component when first deposited resulting in an insufficient number of crystal-nucleation sites.

It seems very unlikely that any combination of these factors would be distributed in territories in developing enamel so peculiar as those of the tufts and lamellae.

The crack-like disposition of the tufts and lamellae has suggested (it seems very reasonably) to nearly all previous workers that they are not present in the enamel when it is first secreted as a very soft, mobile, and adjustable material. However, the crack-like disposition does not necessarily imply a catastrophic development and the little evidence that is available certainly suggests that their formation is by no means sudden.

9.10.

The tufts contain a very significant inorganic component ("interpolation" from the appearances seen in figure 6 A p.169 in BOYDE and STEWART , 1962 would suggest that the tuft regions contain slightly more mineral than dentine) and there is no evidence which would suggest that this is not distributed evenly throughout these regions(the degree of differential argon ion beam erosion etching within the tuft region shown in figures 6A and 6B, p.169 in BOYDE and STEWART, 1962 is no greater than that within the surrounding "normal" enamel). The usual prism and interprismatic domains are found in the tufts. These facts show that the tufts are not catastrophic cracks "between prisms" which have later become filled up with a mainly organic component.

I shall take leave to present the following concept of the development of the tufts, viz:- that they develop in "planes of tension" (ORBAN, 1957) which are occasioned by forces originating in the growth of the crystallites themselves (the forces which would be needed to "squeeze out" a viscous organic matrix during maturation must be very considerable). A reduction in pressure in the regions in which the tufts will develop allows the entry of more organic material which has been mobilised from surrounding regions during maturation. Meanwhile the crystallites continue to grow in diameter in a normal fashion, but they come to be separated by more organic material than in normal enamel. It is not necessary to suppose that the crystallites are of more or less than average diameter.

The main value of any hypothesis is to suggest experiments to test it - the present one suggests that the intercrystalline spacing, crystallite diameter and domain diameters in the tuft regions should be measured.

.1.

C H A P T E R X

SURVEY AND DISCUSSION OF PREVIOUS ELECTRON-MICROSCOPE STUDIES AND RESULTS.

The first electron-micrographs of enamel (two stage polystyrene : silicon oxide replicas) were published by GEROULD (1944) and confirmed the existence of "prisms" in enamel, in the sense that the shape of the "prism-sheaths" in transverse section could be seen. In 1945, GEROULD published more electron-micrographs of silicon oxide replicas of ground and polished, hydrochloric acid etched surfaces. He could distinguish no differences between the enamel rods and interprismatic substance, except that they etched away at different rates. The pattern of orientation of the elements (crystallites and fibres of later authors) in the prisms, which has been figured and described by many later authors as a "feather--like" or herringbone arrangement can be clearly seen in GEROULD's Figure 3A - but he made no comment on this at all.

HELMCKE has claimed (1953, 1958 B) that the first electron--microscopic study of enamel development was undertaken by himself, in collaboration with his colleague KECK, during the war years in Berlin. HELMCKE stated that DR.KECK read a paper entitled "Übermikroskopische Untersuchungen des Aufbaues der Zähne" in Berlin on 24/11/1944, (also cited by FRANK, 1952) which date is in any case later than GEROULD's(1944) publication.

BOYLE, HILLIER and DAVIDSON (1946) examined isolated crystallites from developing human and guinea - pig enamels. They prepared a suspension of scrapings of the developing enamel in collodion; which suspension was cast as a film and examined directly in the electron-microscope. They found the individual crystallites to be 400 - 600 Å wide; to be very long and to give electron diffraction patterns similar to those of hydroxyapatite.

SCOTT and WYCKOFF (1947 and 1949) and MENKE (1950) published electron-micrographs of replicas of polished and etched enamel surfaces, which contained sufficient detail to show the division into prisms. SYRRIST (1949) published replica-pictures showing the

.2. individual crystallites, which, he considered, showed that each crystallite was surrounded by an envelope of organic substance. He found that "the crystallites - as seen in replicas of acid etched adult human enamel- had an average diameter of 1000 Å and lay roughly parallel with the prism axes in the prisms. Similar studies were reported by TAKUMA, SUSUKI, OSAWA and TSUCHIKURA (1949), who found that the crystallites in the interprismatic regions often lay nearly at right angles to the prism direction (cited by SCOTT, 1955) and who also described a submicroscopic fibrillar network (cited by FRANK, SOGNNAES and KERN , 1960).

FRANK (1950 B and C) employed chromium-shadowed collodion replicas and considered that he was able to demonstrate, in these, a network of fine organic fibres permeating the prisms and connected to the organic prism sheaths. FRANK (1952) reported the identification of crystallites in replicas of human enamel.

SCOTT (1952), SCOTT,USSING, SOGNNAES and WYCKOFF (1952), SCOTT and WYCKOFF (1952) and SOGNNAES,SCOTT and USSING (1952) seem to have been the first to have examined ultra-thin sections of enamel; i.e. of decalcified and non-decalcified, developing, methacrylate-embedded material: they also studied replicas. They confirmed FRANK's description of a fine fibrillar network in the prisms and inter-prismatic substance, in direct continuity with the rather more massive organic prism-sheaths. They noted that the organic matrix "becomes fibrillar during calcification" (my italics), and that these "fibrils" become more and more separated as the enamel "approaches maturity". They speculated that this reduction in matrix volume might be effected through dehydration and condensation, rather than by loss of the organic substance itself. They also published electron micrographs of crystalline particles seen in their sections of developing, non-decalcified enamel, but these were probably fragments of larger crystals.

BERNICK, BAKER , RUTHERFORD and WARREN (1952) published some electron-micrographs of (very poor) decalcified sections of enamel. They could not distinguish a separate (organic) interprismatic substance and thought that the prisms were joined together by united prism sheaths.

10.3. KENNEDY, TEUSCHER and FOSDICK (1953) found that the crystallites made an angle of from 20° - 40° with the long axis of the prisms, as a result of their replica study of etched, polished surfaces of human enamel. HELMCKE (1953 "Atlas") published a large number of electron--micrographs of replicas of both fractured, and polished and etched enamel surfaces. He described the feather-like arrangement of the enamel crystallites, i.e. their mutual divergence from an axis within the prism. He found that the crystallites in adjacent prisms abut directly on to each other, without the presence of any intermediate substance and was led to deny (for the first time - often repeated later e.g. 1955, 1956, 1958A, 1960 A and B) the existence of separate prism-sheath or interprismatic regions. HELMCKE (1953) also denied the existence of a membrana limitans at the enamel-dentine junction.

MATSUMIYA and TAKUMA (1954) published another "Atlas", but they showed pictures of thin sections of decalcified and undecalcified developing enamel as well as replicas. They confirmed the existence of the submicroscopic fibrillar network continuous with the prism-sheaths (see also HAJÓSSI, KOHÁRI and BÓNA, 1956 and TAKUMA,KURAHASHI,YOSHIOKA and YAMAGUCHI, 1956). MATSUMIYA and TAKUMA (1954) found the crystallites in the prisms to be almost parallel to the prism axis, and those in the interprismatic substance to have a different orientation.

WATSON and AVERY (1954 A, B and C) studied enamel development in the adult hamster lower incisor and newborn molars in undecalcified, ultra-thin sections of the surface of the developing enamel. In 1954 they were very confused by the prism decussation pattern they found, but by 1954 C they had read TOMES' (1850) paper and were then able to confirm the latter's interpretation of the rodent incisor inner-enamel. Their work is very important for its detailed content and will therefore be considered in detail.

WATSON and AVERY (1954C) found no cell membrane at the inner ends of the ameloblasts and they found that the enamel, as deposited by the ameloblasts, contained crystalline material at all stages of its development. They found then, that the crystals were apparently deposited simultaneously with the organic matrix, and that they increased in thickness as the enamel "matured". They obtained electron diffraction patterns characteristic of an apatite from the

10.4. crystallites, and showed that their c-axes were parallel with their long axes, They described the developing enamel crystals as "ribbon-shaped laths" and they described extremely thin crystals, 30 - 50 Å in diameter. They were not too sure of this identification, however, for they thought that the "fine fibres may actually represent ribbon-shaped macromolecules of eukeratin". They commented that the crystallites within the prisms - " show no preferred orientation for rotation about the long crystal axis, but are oriented quite randomly". The term"wall - material" was adopted for what I would call interprismatic regions (Fig. 1.1.) and "core material" for the prism proper. They wrote that the "inner enamel rods are laid down in two parts, the wall material before the core material. About 10 microns of wall material is laid down to form the open end of the rod. Core material forms at one of the sides of the rod not bounded by a wall and gradually spreads at greater depth from the enamel surface to fill the whole rod. At least in restricted areas, the cores of all rods start on the same side. Walls and cores can be distinguished by the directions of their component fibres". They found that in the "older inner enamel walls are almost non-existent between the rods of adjacent sheets . . . . the cores of rods of adjacent sheets are in contact with one another", in other words, that there is only an interprismatic region between prisms of the same transverse row. "In younger inner enamel" they found 0.2μ thick "pre-core boundaries" (interprismatic substance) between adjacent rows. They forwarded the view, tentatively, that one ameloblast laid down one "wall" and one "core". The crystallites were found to be parallel to the long axis of the prism in the prism (core) and at right angles to the surface of the enamel during its formation in the "wall" (interprismatic regions). They stated that "crossing over of fibres (crystallites) between rods of adjacent sheets is evident". Actually, this crossing over occurs from the interprismatic regions (longitudinal interrow sheet regions of PATTERN 2) between the prisms of one transverse row to that of the next. WATSON and AVERY made the important observation that the surface of the developing outer-enamel was smooth, and that it was hard within 500 Å of the ameloblast cytoplasm. They stated that -"The fact that the surface

10.5. of the forming outer enamel is smooth indicates that rod structure, if indeed there is a rod structure, is not formed in the outer enamel in the same way as that of the inner enamel". They emphasised that the material composing the rod"cores and walls" appeared to be the same, and raised the difficulty for the adherents of the "one ameloblast forms one rod" theory that crystals cross from the wall of one rod,to the next and beyond. Although they could see no cell membranes, they still considered that "perhaps" the "walls" formed extracellularly and the "cores" intracellularly. They presumed that the organic macromolecules of the matrix - less than 20 Å thick, and which neither they, nor anybody else, has "seen" - determined the orientation of the crystals. They found the crystals in rather "maturer" developing enamel to be 5 - 10 μ long and 100 Å x 400 Å thick.

SCOTT (1955, p.575) stated that - "The most fundamental problem in histological studies of enamel continues to be an exact definition of the basic structural unit . . . . Investigations of developing enamel have shown quite conclusively that <u>each prism is laid down by a single ameloblast</u> (my italics A.B.) ..... the prism is the structural unit of enamel". He found that "It is becoming increasingly evident that the prism form most frequently encountered is arcade-like-or scale-like" (p.577), but he also found a -"bizarre configuration which has been seen often enough to warrant mention ... in which well-defined secondary structures seem to be present within the prisms". (These structures, which were called <u>prisms within prisms</u> in Section 2.4.3.1 seem to be the first <u>discovery</u> in enamel, in the geographical sense, accreditable to the electron-microscope). SCOTT also considered that the ..."possibility of fragmentation during sectioning made it unwarrantable " to present estimations of the dimensions of the crystals in sections.

HELMCKE (1955, 1956) and LENZ (1956) concluded that there was <u>no direct relationship between enamel prisms and the ameloblasts</u>, and theorised that enamel development was a purely crystallographic process, controlled by physio-chemical factors in the gel in which hydroxyapatite crystals developed. It should be noted that HELMCKE and LENZ did <u>not</u> base these conclusions on a study of enamel

10.6.

development. They again denied the existence of an interprismatic substance (as did also HAJÓSSI, KOHÁRI and BÓNA, 1956) and so returned to the stand taken for so long by WALKHOFF (1895, etc.). LENZ (1956) compared the organic material in adult enamel to the mortar between bricks.

FEARNHEAD (1957) reported a study of enamel development in rats and stated that - "the electron-micrographs show that the organic matrix of the interprismatic region is formed in advance of enamel prism" - a finding in agreement with WATSON and AVERY (1954) and many light microscope workers, e.g. WILLIAMS (1886), RÖSE (1893), TOMES (1897), PREISWERK (1903), ADLOFF (1914), ESCHLER (1938), WOLF (1942 B) and CHASE (1948). All these workers held that the "Honeycomb" - more or less of it - gives rise to, or is equivalent to, the "interprismatic substance".

LENZ (1957)reported on the cytology of the ameloblasts in human and mouse material. He found a basement membrane between the internal enamel epithelium and the pulp cells and described numbers of mitochondria at both ends of the ameloblasts. As regards the relationship of adjacent ameloblasts to each other, he denied the existence of terminal bars and intercellular spaces - the latter were considered to be artefacts of formalin fixation. In 1959 (A,B and C; and again in 1959, 1960 and 1961)he confirmed these findings and also reported that he considered enamel formation to be an intracellular process. He noted that it becomes very difficult to distinguish between adjacent ameloblasts when their infra-nuclear cytoplasm becomes packed out with endoplasmic reticulum. LENZ's intracellular "fibrils" which he considered to be organic and to have the role of determining the orientation of the"hydroxyl-apatite" crystals - are in fact the inorganic apatite crystals themselves (WATSON and AVERY, 1954 C; FEARNHEAD, 1960 B). LENZ has not been alone in considering the "needle-shaped crystals" (FEARNHEAD, 1961B) to be organic fibres - HELMCKE (1958, 1960A), FEARNHEAD (1957), NYLEN and SCOTT (1958), FRANK (1960) and QUIGLEY (1959 A) have also been adherents of this view.

HALL (1958 - carbon replica study) reported an average crystallite size of 650 Å x 100 Å x 4000 Å in adult human enamel, and found the crystal edges to be serrated with"organic projections on the

10.7. sides of the organic fibres" having a periodicity of 320 Å. SCOTT (1958 - published 1960) and SCOTT and LOSEE (1958) found a 300 Å periodicity of fragmentation of crystallites in carbon positive replicas and pseudo-replicas of ethylene diamine treated enamel. These authors considered that -"since matrix formation precedes calcification the axial fibrils might occur at periodic intervals on the fibrils themselves".

NYLEN and SCOTT (1958) found mitochondria mainly, or only, on the stratum intermedium (supra-nuclear) side of the (mouse) ameloblast nuclei. They also described the remarkable array of rough surfaced endoplasmic reticulum almost filling the infra-nuclear cytoplasm, and the location of the Golgi apparatus just below the nucleus on this side. REITH (1960) found the (rat incisor ameloblast) cytoplasm close to the enamel crowded with secretory granules or vacuoles of various shapes and sizes and paid detailed attention to the morphology of the secretory granules. His four types of granule were all membrane bound, mostly 1 - 1.5 μ diameter, and all found frequently in the Golgi region as well as towards the dentinal end of the cells. Type A contained a light amorphous material filling most of the granules, and also a group of small circular osmiophilic bodies 400 - 500 Å in diameter. Type B had darker contents and no discernable internal structure. Type C contained a dispersion of fine granules and were more opaque than either Types A or B. Type D were the most dense, containing only an opaque amorphous material. REITH called the developing needle-like crystals "rodlets" and could not find any fibrous material in the "matrix". Consideration of the relationship of the ameloblasts to their TOMES' processes or whether enamel formation was intra- or extra-cellular in situation was avoided.

QUIGLEY (1959) also studied enamel development in the hamster, but -" whilst agreeing with the idea of WATSON and AVERY ((1954C)) of a "wall" forming peripherally while the "core" lags somewhat in development, it is felt that the core should be considered a "rod" and therefore the wall would be interrod". QUIGLEY did not find terminal bars but still managed to define the TOMES' processes, because he found that they contained no endoplasmic reticulum. He found no homogeneous precursor substance and thought enamel formation was

O. 8.

intracellular. He found, like LENZ, that it was difficult to distinguish the separate ameloblasts at their (inner or dentinal) distal ends and considered a syncytial nature for this region. His findings are similar to those of WATSON and AVERY with regard to the formation of the rods:- "Alternate layers of cross sections of rods between longitudinal sections show that the interrod matrix forms first (my italics A.B.). The interrod material forms all around the as yet cytoplasmic rod except on one side. This open side, always on the same side of the core in a given sheet or layer or rods, eventually closes, but just prior to its completion, fibrillation (i.e. crystallite growth A.B.) of the rod commences from the open side..... When the rods are fully fibrillated, the only difference between rod and interrod substance is in the direction of the fibrils. In the incisor, there is more interrod substance between two rods of the same sheet than between rods of adjacent sheets". QUIGLEY (like WATSON and AVERY) could not find a structure identifiable as a prism sheath. He dismissed possible differences between the hamster molar and incisor enamel with a comment that they appeared to be the same "in general". QUIGLEY (1959B) found intact cell membranes at the dentinal end of the hamster ameloblasts until the formation of enamel commenced. He considered that -"The breaking up of the double membrane at the basal end of the ameloblasts would allow a limited penetration of enamel by the dentin, and would account for the formation of the so called enamel spindles". He also saw his failure to demonstrate distinct cell membranes between the dentinal ends of adjacent ameloblasts as evidence against the"one ameloblast forms one rod" theory.

FRANK (1957) gave an average length of 7,500 - 10,000 Å for the hydroxyapatite crystals in human enamel. In 1959 he gave his interpretation of the appearances seen in his ultra-thin sections of non-decalcified mature human enamel cut with a diamond knife. He considered that his electron-micrographs revealed the position of an organic reticulum in and between the enamel crystals. I am opposed to his interpretation on the grounds that the image lines he points to could equally well be caused by cracks in,and between, the crystals. I cannot see how it is possible to bend these crystals through more

10.9.

than 60° without fragmenting them. FRANK's (1959) fibrillar matrix was supposed to be "made up of a fine three-dimensional reticulum forming round honey-combed meshes of 115 Å diameter"; to be both intra- and intercrystalline, and to be continuous through the prism, prism-sheath, and interprismatic substance. FRANK described a "penniform" arrangement of the crystallites within the prisms (as HELMCKE , 1953, 1955, etc.) and found that the longitudinal axes of the crystallites lay at angles between 0° and 45° to the long axis of the prism within the prism and at 0 - 90° in the interprismatic substance. These measurements of the crystallite orientation were made directly on the electron-micrographs and so, of course, only contribute two-dimensional information to a three dimensional problem. FRANK (1959) claimed to be able to detect variations in the degree of mineralisation of the enamel in his sections ! FRANK's (1959) views of the nature and situation of the organic matrix in adult enamel were re-iterated in papers by FRANK and SOGNNAES (1960), FRANK, SOGNNAES and KERN (1960) and FRANK (1961), but these authors also described how the structure develops in human and rat enamel - "It seems quite clear from our non-decalcified sections of calcifying and calcified enamel that apatite crystallisation starts specifically on and within the longitudinally oriented protein fibrils of the pre-existing reticulum matrix" (FRANK and SOGNNAES, 1960, p.346). These latter authors recognised the decussation of the rat incisor inner-enamel prisms. They stated that-"From our results, it seems that within the middle enamel layer (inner-enamel of TOMES (1850) A.B.) the cytoplasm of one ameloblast contributes to the elaboration of more than one enamel rod". They make reference to their Figure 13, but fail to explain how they interpolated to this conclusion. I have mentioned it, however, as the first mention of this opinion by "electron-microscopists", and because I have been led to this as a general conclusion for (PATTERN 2 and PATTERN 3) mammalian enamel development. Their "inner enamel" is the layer only 0.5 - 2 μ wide in which all the crystallites are parallel. In rat molar enamel, FRANK and SOGNNAES (1960, p.345 and Fig. 18) found that - "apatite crystal orientation may be at right angles from one rod to the next". FRANK et al considered enamel formation to be an intra-cellular

10.10. process, as did also PLACKOVA and STEPANEK (1961), SCOTT and NYLEN (1960) and NYLEN and SCOTT (1960).

SCOTT and NYLEN (1960) and NYLEN and SCOTT (1960) describe the (electron-microscopic identification of) terminal bars at the stratum-intermedium end of the ameloblasts, but not at the dentinal end of these cells. They considered that the structures which had been identified with the light microscope as terminal bars at the inner (dentinal) ends of the ameloblasts, and "which in the generally accepted concept of amelogenesis" give rise to the interrod-substance, were in fact -"Extracellular material identical in character to young enamel matrix. The identification of these deposits ....effectively dismisses the questions and problems that have arisen as a result of their interpretation as terminal bars". These authors also described typical desmosomes at "various points along the laterally contacting basal ends of the ameloblasts, as well as along the line of contact between the ameloblasts and the cells of the stratum intermedium". They also described a process of segmentation of the ameloblast: a repetitive budding-off of the TOMES' process of rat incisor ameloblasts, which would account for the cross-striation of the enamel prisms. They considered the interrod substance to be formed first and extracellularly; the rod-substance intra-cellularly, i.e. within the detached piece of ameloblast. SCOTT and NYLEN (1960) also recognised the thin layer of material at the enamel-dentine junction apparently continuous with the inter-rod substance and described by FRANK and SOGNNAES (1960). They thought that this layer corresponds to the dentino-enamel membrane of earlier authors: I agree with this opinion. They still considered that the orientation of the mineral component was determined by the configuration of the organic matrix, which they believed to be fibrillar at the onset of matrix production.

FEARNHEAD (1960 A and B, 1961 A) found complete cell membranes at the dentinal end of rat ameloblasts, in carefully Araldite-embedded material, and thus showed that enamel formation was entirely extra-cellular. WATSON (1960) confirmed this observation almost simultaneously, and credited his success to the use of "carbon--sandwiched" sections. FEARNHEAD believed, at this time, that "fibrillogenesis occurs extra-cellularly, the fibres forming from a

10.11. granular precursor substance which is elaborated and secreted by the ameloblasts". WATSON called FEARNHEAD's "extra'cellular granules" - "stippled material". FEARNHEAD (1960 B) showed that diffraction patterns, characteristic of hydroxyapatite, could be obtained from the region of the "formative front" of enamel containing the very fine (50 - 100 Å diameter) electron-dense "fibres", and he therefore concluded that the latter are, in fact, the newly deposited enamel crystallites. He also suggested the name "mineralising front" for this region. It is interesting to note that all other workers up to this time (except WATSON and AVERY, 1954C) had accepted these needle-like crystals as organic fibres. FEARNHEAD (1960B) suggested, that although one could only presume the position of the organic matrix to be that of the electron-transparent intervals between the crystals, this did not rule out the epitactic mineralisation concept. WATSON (1960) raised the query whether the demonstration of the extracellular site of enamel formation would question the eukeratinous nature of the organic matrix.

FEARNHEAD (1961B) thought that the terminal bar apparatus -"is apparently a condensation of stainable material between the ameloblasts, in a position which marks the boundary between the cell and its 'TOMES'process' ". FEARNHEAD (1960C, 1961B)described and figured discrete lengths of thickened cell-membrane surrounding (apparently recently secreted) accumulations of extra-cellular granular material. From FEARNHEAD's work it is clear that these thickenings are not to be confused with the characteristic thickening of adjacent cell-membranes seen in desmosomes and terminal bars. FEARNHEAD (1960, 1961B) still considered that the orientation of the inorganic component depended on that of the organic fibres, which he considered might be formed from the fusion of the adjacent 50 - 70 Å granules of the "extra-cellular granular material".

FEARNHEAD (1960B) had described the most mature developing rat enamel crystallites that he had studied on that occasion, as "tape-like". JOHANSEN and PARKS (1961) called the equivalent crystallites in developing human deciduous enamel, "plate-like" structures. HÖHLING and ERWIG (1960) and HÖHLING (1961) described truly hexagonal, transverse-sectional shapes for the crystallites of

10.12. adult human enamel seen in carbon replicas.

...The first electron-microscopic study of the cross-striations of the prisms (in serial replicas of acid etched human enamel) was that of HELMCKE, SCHULZ and SCOTT (1961, 1963; and personal communication from J-G.HELMCKE, 1962) - -"Gentle constrictions were noted along the prisms at the same regular intervals as those assumed by striations. In addition, certain characteristic arrangements of the ultrastructural components were seen within prisms. In the central prism core the crystals and organic elements were aligned fairly parallel to the prism axis,while in the cortical region they tended to incline outward, angulation being greatest in the widest portions of the prisms" (HELMCKE, SCHULZ and SCOTT, 1961 - see discussion Section 8.3.).

LENZ (1961) provided confirmation of the higher organic content of the tufts , from a study of decalcified human enamel with the electron microscope.

REITH (1959, 1961) has described the changes in the cytology of the ameloblasts at the commencement of maturation in the rat incisor.

Recent Publications on Electron Microscopy of Amelogenesis.PANNESE (1960, 1961, 1962) has reported a very thorough study of the enamel organ of the cat. His findings are mostly concerned with the stages before and after amelogenesis. PANNESE (1962) considered that "exchanges of material occur through both the internal and external enamel epithelia" in the stages prior to dentinogenesis - "But the exchanges through the latter epithelium predominate, for the external enamel epithelium is thinner and closer to the capillaries in the mesenchyme". He also found that the odontoblasts are connected to each other (at their outer ends) by typical terminal bars, and to the inner enamel epithelium by typical desmosomes. (STUDNICKA (1917) had described "Cytodesmen" in this situation). PANNESE described the development of the mutual interdigitation of the ends of the inner enamel epithelium cells and future odontoblasts during the stage of the first differentiation of the inner enamel epithelium, when the basement membrane of electron microscopists ("a submicroscopic lamella of 100 - 300 Å in thickness, which is electron-dense after OsO_4

10.13. fixation") has already disappeared. PANNESE considered that there are intercellular spaces between the inner enamel epithelial cells, which develop during the stage prior to their final differentiation into ameloblasts, and which are continuous with the intercellular spaces of the stratum intermedium and, hence, the stellate reticulum. PANNESE did not mention the fate of the desmosome-like attachments between the odontoblasts and inner enamel epithelium at the commencement of amelogenesis, nor did he comment on the presence of terminal bars at either end of the inner enamel epithelial cells (or later, ameloblasts).

PANNESE (1960) considered that "the characteristics of the Golgi complex, the abundance of microvesicular structures in the cytoplasm and the presence of microvilli on the cell surface" indicated that the stellate reticulum and stratum intermedium cells "probably perform a moderate secretory activity" and that -"It can be assumed that in this way an intercellular material is produced which occupies the large intervals in the stellate reticulum".

RÖNNHOLM called the inner and outer terminal bars (and associated terminal web) "distal and proximal ring-shaped septa". He considered that (1962,A.p.242) "these ring-shaped septa certainly correspond to what has been described earlier (i.e. by light microscopists, A.B.) as the terminal bars of the ameloblasts", but that the "distal septum, does not appear structurally identical to what has been described as terminal bars in different types of epithelia". RÖNNHOLM confirmed the observation of FEARNHEAD (1960) and WATSON (1960) that enamel develops extracellularly. He showed that (1962, B. p. 256) "the enamel crystallites are found together with filaments with cross banding characteristic of collagen" at the beginning of amelogenesis and that (1962, B.p.258)-"the enamel crystallites to some extent lose their characteristic orientation at the contact zone between enamel and dentine". He showed clearly that the development of prisms in the enamel is associated with the appearance of the TOMES' processes, after a narrow seam (ca.1.5μ) of enamel containing crystallites oriented predominantly perpendicular

10.14. to the enamel-dentine junction had been formed. "The boundary between the ameloblasts and the newly formed enamel as observed in longitudinal sections" (i.e. the Picket fence) was described as having a "jagged serrated appearance, with each ameloblast cell forming one tooth in the saw edge-like boundary" (1962, B.p.262). RÖNNHOLM (loc. cit.) made much of a process from each ameloblast "which partially covers the TOMES' process of the neighbouring cell". It appears that it is principally on these grounds that he concluded (1962 B.p.270) "that each ameloblast cell is topographically related to at least two prisms and that the interprismatic gap is not topographically related to the boundary between the ameloblasts". My view differs from RÖNNHOLM's in that I have only occasionally seen the appearance which he regarded as showing the existence of a process covering the neighbouring cell, but I still regard it as evident that one prism may be contributed to by two (in PATTERN 2 enamel development - Fig. 2.11.) or three(in PATTERN 3) ameloblasts. RÖNNHOLM did not report on the appearances seen in other than longitudinal sections and although he did not attempt to give a three-dimensional picture of the relationship of the ameloblasts cell surface and the orientation of the enamel crystallites, his concept is necessarily considerably at variance with mine because he did not recognise interprismatic regions - his "interprismatic gaps" correspond to my "prism-sheath" or "prism-boundary" planes. He believed that these "gaps" are <u>real</u> structures which appear after a first stage in which the prisms appear to be closely packed, but that they disappear again later. I believe that they are most likely to be embedding artefacts.

RÖNNHOLM correctly identified the crystallites (after FEARNHEAD). However, he believed that they have a limited length (of about 910 Å at the ameloblast cell membrane; 1180 Å just 1 - 2 μ away; and in the adult tissue, 1600 Å). What FEARNHEAD, WATSON and AVERY (1954) and myself (among others)regard as single, extremely long, crystals he considered to be rows of crystals placed end to end. These rows showed a "<u>staggered</u>", overlapping arrangement. WATSON and AVERY (1954,C), FEARNHEAD and myself, all refer to single crystals in which there is not the slightest sign of staggering, or overlapping, i.e. no indication that the "single crystal" is really a row (other than that

10.15. they may behave as a sort of row in diffraction patterns, or appear as a sort of row of lighter and darker bands in light and/or dark field images because parts of their length may - and other parts may not - satisfy the BRAGG diffraction conditions for relevant crystal planes). These crystals may be isolated from developing enamel by preparing a suspension (collodion - BOYLE, HILLIER and DAVIDSON, 1954; or in water FEARNHEAD, 1960) and "single crystal diffraction patterns have been obtained from (these) 'tape-like' crystals showing that there are no major discontinuities within the crystal lattice"(FEARNHEAD and ELLIOTT 1962).

RONNHOLM (1962B) described the "plate-like" crystals as being arranged in groups "with both their long axes and their broad surfaces in parallel.... The crystallites in different groups are rotated at various angles around their long axes". This seems to be the first accurate description of this arrangement; I have confirmed it in all the mammals that I have studied. The emphasis on this statement can be changed by saying that there is some degree of preferred orientation of the a-axes of the crystallites, but only over limited regions.

RONNHOLM (1962, B. p. 274) believed that "simple geometrical considerations" determined differences in contrast in the electron-micrographs of crystallites. Thus, where the long-axes of the crystallites (rows of crystallites end-to-end for RONNHOLM) lay in the plane of section - "when confining measurements to crystallites that appear with highest contrast, the measurements (of crystallite "thickness" i.e. narrow diameter A.B.) will be confined to crystallites that are orientated with their broad surfaces roughly parallel to the electron beam". FEARNHEAD and ELLIOTT (1962) have shown that variations in the contrast in bright-field images of single crystallites can occur, and that dark "extinction regions" can move along the crystal when it is tilted through a few degrees in the electron-microscope. In addition, any image of transversely sectioned crystallites will reveal great variations in contrast amongst the individual crystallites. It is therefore, considered that RONNHOLM's deliberations on the crystallite orientation and contrast question are too elementary.

RONNHOLM (1962 B. p. 279) wrote that "during the earlier stages of crystallite growth the crystallites show a spindle shape".

10.16. This may really be so, but a more careful consideration of the evidence is warranted, since : 1) it would be expected that obliquely "sectioned" crystals would appear to taper at their ends and: 2) it could only be shown that the crystallites are not obliquely sectioned by an elaborate stereoscopic technique and: 3) out-of-focus "artefacts" and extinction-contour "artefacts" ought to be excluded.

RÖNNHOLM confirmed the observation that new crystallites only form at the mineralising front . His studies of the increase in diameter of the crystallites with increasing distance from the "front" are interesting (they are considered in 3.4.) but, because of criticisms of the nature just raised, it is necessary that they be repeated and extended. RÖNNHOLM (Thesis, 1962, p.7.) studied the crystallite thickness (smaller diameter) in different parts of the enamel , and found that "the crystallites are thicker at the free surface of the enamel and thinner at the dentine-enamel junction". RÖNNHOLM's (1962 B) measurements were made (in this case) on diamond knife sections of "adolescent" teeth. This material must be considered very suspect - he provided no evidence that the regions compared had reached the same degree of mineralisation - and, further, the captions and text referring to the histograms (compare captions to charts 6 and 7 on pp. 278 and 279 with text on p. 281) are difficult to follow, and apparently self contradictory. If this particular result of RÖNNHOLM's can be confirmed, one of its possible implications might be that each ameloblast can only "nucleate" a particular number of crystals, as would be the case, for example, if the crystallites or the organic matrix on which they grew by epitaxy were extruded through a constant number of holes in a sieve-like ameloblast cell-membrane. This seems as unlikely as RÖNNHOLM's view that the increase in prism diameter might be mediated via the increase in crystallite diameter.

RÖNNHOLM (1962 B) concluded that the formation of the organic matrix - which like FEARNHEAD (1960) and WATSON (1960) he identified with the stippled material - and calcification (i.e. crystallite formation) appeared to start "more or less simultaneously", but he nevertheless lent his support to the view that the crystallites formed on the surfaces of the "septa" of the organic "stroma" (RÖNNHOLM,1962C).

10.17. I am unable to understand how he identified (in 1962 B,p.298) the "fibrillar elements of enamel (which) are soon embedded in a granular ground substance" described by FRANK, SOGNNAES and KERN (1960, p.173 - which these authors considered to be formed intra-cellularly) with the tonofibrillae of the inner terminal web of the ameloblast cytoplasm (" distal fibrous ring structure" - RÖNNHOLM , 1962 B.p.298).

RÖNNHOLM correctly criticised the view of SCOTT and NYLEN(1960) that portions of ameloblast are separated off (to form cross-striation segments of the enamel prisms), but he erred in explaining how these authors misinterpreted their micrographs. He stated that (1962 B. p.298) -"A part of each ameloblast cell extends over and covers a part of the distal surface of the adjacent cells. This lateral extension of the cell starts from the terminal bar region. A section cut through this extension in certain orientations may make the extension appear to be separated from the proximal part of the ameloblast cells by a cell boundary".

RÖNNHOLM held - like WALKHOFF (1895, etc.) - both that there is no interprismatic substance and that each prism is surrounded by a less-mineralised cortical zone. He supposed this zone to develop in relation to the sides of the ameloblasts and to contain crystallites which are "orientated more or less perpendicular to the axis of the prism .....In the region where the orientation of the crystallites is less precisely parallel than in the rest of the prism this less precise orientation would result in a lower degree of calcification because less perfect orientation results in larger interspaces between the crystallites and more space for matrix material between the crystallites"(1962 B. p.299). He used the same argument as many earlier workers to support his contention that there was a more organic edge-zone; i.e. that (he found that) this zone appears elevated above its surroundings (in replicas of) acid-etched ground surfaces. However, there is no evidence that differential acid-etching rates are related to the organic: inorganic content ratio rather than to differences in the orientation of the crystallites.

FRANK and NALBANDIAN (1962) and NALBANDIAN and FRANK (1962) have described the appearance of the prisms filling in from one side (Section 2.4.2.3.). They state that -"After a certain length of

10.18 interrod substance is formed, there is a progressive filling in of the future rod area. In(the) case of arcade-formed enamel(the)rod matrix forms first on the open side of the arcade. In the early stages calcification is more advanced in the interrod substance than in the core of the rod" (FRANK and NALBANDIAN 1962, p.431). They also state (p.422) that - "In human amelogenesis .... a given rod appears to remain in close contact with a particular ameloblast throughout enamel formation. On the other hand, in the rodent incisor it is not certain that a group of ameloblasts retains a constant relationship with one enamel region because of the continuous eruption of the tooth". This argument would seem to deny the movement of the ameloblast layer with the forming enamel and cannot, therefore, be accepted. FRANK and NALBANDIAN also described the appearances called Prisms within Prisms and Spiral Prism Sheaths in Section 2.4.3.1. They confirmed the view (of NYLEN and SCOTT, 1958) that -" In the ameloblast, the mitochondria are located between the nucleus and the non-formative end of the cell, separated from the endoplasmic reticulum, which is arranged in long parallel rows at the formative end of the cell". [I have found elongated , sausage-shaped mitochondria arranged parallel with the α-cytoplasmic membrane array in the inner part of the ameloblast. Their presence in this situation does not seem to have been commented on by previous workers.]

Thus the only works which have appeared since 1961 and deal with the precise aspects of amelogenesis with which I have particularly concerned myself have been those of RÖNNHOLM (1962,A,B), NALBANDIAN and FRANK (1962) and FRANK and NALBANDIAN (1962). However, several papers communicated to scientific societies have had as their principal concern the relationships of the organic and inorganic components during amelogenesis. The field might be surveyed very briefly by saying that FEARNHEAD (1963,A,B) has been led to cast severe doubts on the existence of a fibrillar organic matrix prior to crystallite-genesis; that he has been widely opposed by everyone else in the field (particularly FRANK: FRANK and NALBANDIAN, 1962, 1963; FRANK, WOLFF and GUTMANN, 1964), but that he now receives a more generous hearing and has even been supported by SCOTT (1962) who said -"Although there is no doubt that collagenous matrices are

10.19. fibrillar prior to the influx of inorganic salts, evidence is accumulating which supports the earlier concepts (SCOTT does not say whose concepts) that in some tissues, such as enamel, the original matrix may be amorphous, becoming structural as the inter-crystalline spaces diminish during mineralisation".

FEARNHEAD tried to produce "fibres" in the developing enamel front region, using a variety of techniques, and without any success (FEARNHEAD and ELLIOTT,1962; FEARNHEAD, 1963 A,B). FEARNHEAD and ELLIOTT assumed that"enamel crystals are not deposited on to a fibrous matrix and that the fibres observed in decalcified preparations are produced by the fixation and decalcifying procedures". I am strongly inclined to accept the view that the organic "fibres" seen in decalcified sections of the developing enamel front are only the remnants of the organic matrix packed and ordered into this disposition between, and by, the crystallites themselves.

Other workers have not been inclined to make the complete volte face which FEARNHEAD has done. It is obvious that some earlier workers (e.g. LENZ) had confused the enamel crystallites themselves with the mythical organic "fibres". FEARNHEAD (1961 B) described ordered rows of the 50 - 70 Å diameter "granules" in the amorphous secretory product of the ameloblasts lining up to form fibres. FRANK and NALBANDIAN (1962) claim to have seen real fibrils in this very same (amorphous) region, although they are not reproduced in their illustrations. They stated that (1962 p.429) -"Although the enamel matrix at this stage has a fibrillar appearance, individual fibrils are not easily resolved, because of the electron-dense ground substance. ....This difficulty, encountered by NYLEN and SCOTT (1960) and FEARNHEAD, can also be explained by the very minute size of the fibrillar elements". The "electron-dense ground substance" to which they refer is presumably the remarkably low electron-scattering (in contrast to the enamel crystallites) material between the enamel crystallites, which is continuous with the extracellular granular material of FEARNHEAD (amorphous material: i.e.the stippled material of WATSON (1960)).

EASTOE apparently finds no objections on structural chemical grounds to the idea of a rather mobile enamel matrix. In fact, he stated that (1963, p.647) -"The behaviour of enamel matrix can be more

10.20. readily accounted for by assuming that the protein molecules form a concentrated amorphous gel structure rather than a highly oriented assembly of fibres (FEARNHEAD, 1963). The possession of thixotropic properties by the gel would increase its potential mobility and facilitate an economy of material since the protein would flow from regions where rapid growth of apatite crystallites caused a local increase in pressure to adjacent, relatively unmineralised regions, where it could initiate further crystals".

FRANK and NALBANDIAN did not agree with HELMCKE that the rods, rod-sheaths and interrod substance do not exist in human enamel. They wrote (1962,p.432) -"Of course, in the adult tissue, these three elements are sometimes difficult to differentiate because of the high and relatively uniform degree of calcification. But, with gentle artificial decalcification, it is always possible to visualize, once again, the classic structures previously described, though fine details may be altered by the decalcification procedures". NALBANDIAN and FRANK (1962) considered that the interprismatic substance is more calcified than the prism-substance, and that it contains crystallites whose axes diverge markedly from those of the prism. The crystallites in the prism, on the other hand, are either parallel with the prism, or diverge from it in a feather-like configuration. Their views concerning the crystallite orientation agree in essence with my own, but their views on the difference in degree of mineralisation of prism and interprismatic regions are based on electron-micrographs of demineralised sections and deserve to be treated with a certain degree of distrust.

FEARNHEAD and ELLIOTT (1962) and NYLEN, EANES and OMNELL (1963) confirmed that the "needle-like particles" in the "mineralising front" are indeed apatite crystals, and that their c-axes parallel their long axes. RÖNNHOLM (1962), SCOTT and NYLEN (1962) and NYLEN and OMNELL (1962) have published micrographs showing lines bisecting the images of developing enamel crystallites. NYLEN, EANES and OMNELL (1963) reported that this line disappears at exact focus and suggested "that it may be an interference pattern due to a phase discontinuity in the crystal", - perhaps a twinning plane. SCOTT and NYLEN (1962) and NYLEN and OMNELL (1962) have shown parallel striations with a

10.21. periodicity of 8.2 Å , parallel to one of the three pairs of edges of cross cut developing enamel (apatite) crystals. NYLEN and OMNELL (1962) stated that - "The high degree of regularity of the striations across the entire face of each enamel crystal excludes the possibility of structural imperfections and thereby the presence of organic fibrils within the crystals. In mineralised specimens the apatite crystals were the only components visible . In decalcified sections, however, thin membranous structures were seen which conformed to the morphology of the cross-cut hexagonal crystals and no other structures were observed within or between these configurations. These observations suggest that .... at least part of the matrix is in such intimate contact with the crystal surfaces that it cannot be resolved microscopically in non-decalcified specimens". They also suggested that the apparent "emptiness" of the spaces between the crystals in sections of developing enamel might be accounted for by the loss of the (50%) soluble protein fraction during processing the tissue.

HELMCKE and RAU (1962) confirmed the well known decussation pattern of the prisms in rat and mouse incisor enamel. They could not detect any cross-striation in the fibrillar bundles (prisms) which they described. However, HELMCKE (1963 B)reported finding the cross-striations in Murine enamel, though he gave no further details. He also reiterated the observations of HELMCKE, SCHULZ and SCOTT (1961 and 1963) concerning the nature of the cross-striations in human enamel prisms. HELMCKE (1963A) reported some very interesting observations on the crystallite orientation in enamel which had developed in transplanted rat incisor tooth germs. These tooth germs were removed (by the late Prof. H.S. Fleming) to sites in the eye and brain at an early state of development. Enamel developed - but the regular repetitive pattern of crystallite orientation was found (by HELMCKE) to have gone markedly astray. I have seen HELMCKE's original micrographs, which do him infinitely greater justice than the published reproductions, and can say that much structure that I regard as real and the common property of mammalian enamels is still present. That is, the enamel structure can still be described in terms of three-dimensional domains in which the crystallite orientation changes but gradually- which domains abut against each other at "discontinuities" in the gradual change in

10.22 crystallite orientation (the prism-sheaths). The change in environment of the tooth germ has, apparently, only affected the pattern in which the "fields" formed.

Although SCOTT has lent his name to the ideas of HELMCKE on the nature of the cross-striations of the enamel prisms (see HELMCKE, SCHULZ and SCOTT , 1961 and 1963), he still seems to believe that they are formed by a process of segmentation of the ameloblasts (NYLEN and SCOTT ,1960; SCOTT and NYLEN, 1960) for in (SCOTT) 1962 we find - "Although more work is needed it seems correct to consider enamel matrix formation as extracellular, because even though the interprismatic portion is produced by conversion of distal segments of the ameloblasts, the cytoplasm involved is first isolated by development of new terminal cell membranes".

BRAUER (1962) has found the same elements in his electron-micrographs of replicas of fossil, tertiary mammalian enamels (Creodonts) as are found in recent material (see, for example, HELMCKE 1953, 1955, etc.).

Micro-x-ray diffraction methods have not yet been widely applied to the study of mammalian enamel. CARLSTRÖM(1960) has provided a notable review of the method, and published a microdiffraction pattern obtained from a "single enamel prism" measuring 6 x 6 x 50 μ. He noted a well preferred orientation of the apatite crystallites; their c-axes being parallel to the long dimension of the enamel prism.

GLAS (1962) reported some very careful micro-x-ray diffraction studies of the orientation of the crystallites in a single bundle of human enamel prisms. He confirmed that the c-axes of the crystallites lie essentially along the course of the prisms, but that they deviate: the majority not more than 30 - 40° in any direction - the maximum deviation found was 73°. He provided diagrams showing the proportion of crystallites making various angles with the prisms in four zones along his one bundle. He found that the mean deviation was always less in a mesio-distal plane (equivalent to my "Battlements" plane - 2.4.2.3.- Diagram 2.12) than in a sagittal plane (equivalent to my "picket-fence" plane - 2.4.2.3. Diagram 2.11) and that this asymmetry was most marked in the central portion of the enamel. These results are entirely in

10.23 agreement with the present findings (2.4.2.3.). GLAS found that the a-axes of the enamel crystals are randomly oriented.

ANGMAR, CARLSTRÖM and GLAS (1963) reported the ingenious use of micro-x-ray diffraction to determine variations in the Ca: P ratio in ground sections of adult human enamel. They stated that if the Ca:P ratio is higher than apatite (Ca: P, 2.16) then lines of CaO appear in the diffraction pattern after heating to 700-900°C. Usually the Ca:P ratio is of the order of 1.94 , i.e. less than that of apatite, when lines characteristic of $Ca_3 (PO_4)_2$ appear under the same circumstances. They detected a slightly lower Ca:P ratio in outer enamel than in inner enamel by these means. These authors also found that inner enamel had the lowest degree of preferred orientation of its apatite crystallites: "Close to the dentine-enamel junction the orientation was often very poor, as indicated by the bending and twisting of the enamel prisms in this area".

11.1.

C H A P T E R XI

GENERAL SUMMARY AND CONCLUSIONS.

Enamel development has been studied in a number of mammals by various techniques, including:- electron microscopy of ultra-thin sections of the developing enamel and ameloblasts : light microscopy of decalcified, stained sections of developing teeth : the preparation of wax reconstructions, showing the shape of the surface of developing enamel, from serial $\frac{1}{2}\mu$ thick sections (from the blocks of tissue prepared for electron microscopy): scanning (reflection) electron microscopy of the surface of developing enamel : the analysis of the mineral content in "maturing" enamel by scanning electron probe x-ray emission microanalysis and tetracycline antibiotic fluorescent "labelling" of the developing enamel.

The developmental studies have confirmed that enamel development occurs extracellularly and that at least a thin layer of extracellular granular material lies between the ameloblast cell membrane and the "mineralising front" of the enamel.

The basic features of enamel structure can be considered in terms of a gradual change in orientation of the structural elements (i.e. the crystallites and between them, an organic matrix which may be fibrous or not) in three dimensional domains , and the boundary planes (prism sheaths) occurring at the abutment of adjacent domains.

Most enamel crystallites seem to develop with their long (c-, hexagonal-optic-,unique-) axes at right angles to the nearest part of the surface of the developing (mineralising) front, except where there must be assumed to be a relative movement between the mineralising front and the adjacent ameloblast - (TOMES' process) - surface. The complex pattern of orientation of the crystallites in the developing (and adult) enamel is, therefore, related to the complexities of the shape of the developing front. The shape of the developing front of the enamel is presumed to be related to the shape of the secretory poles (TOMES'processes) of the ameloblasts and this is itself, presumably, the result of a dynamic equilibrium between the(pressures or forces in the) cells and their secretory product.

11.2. The existence of the TOMES' processes - and hence a repetitive crystallite orientation pattern - depends upon a certain degree of secretory activity (pressure) on the part of the ameloblasts. At the very beginning and the very end of enamel development there are no TOMES' processes; there is therefore no complexity in the crystallite orientation pattern because they all tend to lie at right angles to the almost plane surface of the mineralising front and the inner ends of the ameloblasts ; and there are therefore no prism-sheaths and, therefore, no prisms in the innermost and outermost layers of enamel.

The hypothesis is put forward that the cross-striations of the enamel prisms (shown by HELMCKE, SCHULZ and SCOTT (1963) to be due to a periodic change in crystallite orientation along the prism axes) are caused by periodic changes in the shape of the mineralising front, related to the circadian (about 24 hour) rhythm of secretory activity of the ameloblasts.

The shape of the developing enamel surface and its relationship to the "prisms" has been studied in detial. Three significant planes of section are described. The"Picket Fence" appearance of the enamel surface occurs in sections which contain the longitudinal axes of both the prisms and the ameloblasts at that level. The "Battlements" appearance is seen in a plane of section at right angles to the previous one, but which still contains the long axes of the prisms. (The "Boxes" appearance occurs in sections containing a plane at right angles to the first, and containing the long axes of the ameloblasts - which more often lie at an angle of the order of 45^{o} to their prisms. The interpretation of the "Boxes" as a manifestation of segmentation of the ameloblasts, in order to account for the development of the cross-striations, is shown to be erroneous). The "Honeycomb" appearance occurs in tangential sections of the developing enamel surface.

In most mammals, the holes in the"honeycomb" (depressions in the (developing) mineralising front) fill in from the one side contained in the angle between the ameloblasts and their "prisms". There is no discontinuity between the "interprismatic" and "prism" regions on this side (i.e. no prism sheath).

11.3. Each hole in the "honeycomb" of the developing enamel is occupied by one ameloblastic process. The secretory territories of ameloblasts are not necessarily equivalent to prisms in the formed enamel. One ameloblast may contribute precursor substances to three prisms in PATTERN 3 (Fig. 1.3) enamel formation ; to two prisms in PATTERN 2 enamel formation; but the central part of the secretory territory of one ameloblast is equivalent to one prism in PATTERN 1 enamel formation.

The prisms in alternate zones (lamellae, layers, dia- and para-zones , HUNTER -SCHREGER bands, or pseudo-prisms of KORVENKONTIO, 1934 - 35) fill in from alternate, not exactly opposite, sides. This is most dramatically shown in the rat incisor, where each zone consists of one layer of prisms.

Decussation of the prisms is absent in the enamels of the members of the orders Sirenia, Cetacea, Insectivora and Cheiroptera which have been examined.

Arcade or Horseshoe-shaped prism cross-sections predominate in the orders Primates and Proboscidea. The presence of more "interprismatic substance" leads to the slightly more hexagonal horse-shoe outline with a greater frequency of complete prism sheaths in the Carnivora. A greater preponderance of interprismatic substance in the Sirenia, Cetacea, Insectivora and Cheiroptera is associated with the occurrence of almost round prisms with more, complete prism-sheaths. Ovoid cross sections are very common in the Ungulata and are associated with the presence of a great deal more interprismatic substance between adjacent longitudinal rows of prisms than between prisms in the same longitudinal row. The formation of longitudinal rows (as against the transverse rows of the zones or HUNTER -SCHREGER bands) of prisms in Ungulates is particularly prominent. This feature is also found in Lagomorphs and Marsupials and much less in the Primates, Carnivores, etc. It is well marked in the Rodents, but often well masked by the decussation of the narrower zones in this order. (Where the row formation is not marked in the Ungulata, the tendency is towards the round cross-section with abundant interprismatic substance of the Cetacea, etc.). The rounded-off rhomboid shape of rodent incisor inner-

11.4. -enamel prisms (particularly in the Muridae and Sciuridae) is associated with the decussation of alternate transverse rows of prisms.

Special attention has been given to the question of the origin of the "tubules" or "fibres" in marsupial enamel. They are not dentinal formations; but regions in which crystallite formation does not occur.

The ameloblast cytoplasmic architecture is described. The presence of scattered RNA particles in the Stratum Intermedium cells would suggest a protein synthetic function, but if this protein or any other product of the Stratum Intermedium is associated with amelogenesis, it is not passed through or between the ameloblasts in the form of discrete secretion granules.

The concentration of mineral in developing enamel was studied by measuring the Ca K_α emission under electron bombardment (scanning electron probe x-ray emission microanalysis); and was found to increase almost linearly with distance from the ameloblasts. There is no sudden increase in the rate of increase in concentration, as proposed by many earlier workers. (ROSSER, BOYDE and STEWART, 1964).

Structural observations have been made using the established techniques of light and electron microscopy, and the newer techniques of x-ray emission microanalysis and ion beam erosion and etching, and secondary (ion excited) electron emission microscopy.

The disposition of the HUNTER-SCHREGER bands (or zones) in the cuspal enamel of Carnivores (and man?) is a spiral. The centre of the helix is represented by the gnarled enamel of the cusp tip. All the other appearances of (the arrangement of) the HUNTER-SCHREGER bands are explained by this disposition. The HUNTER-SCHREGER bands appear in the light-microscope image of enamel because the scattering of (blue) light in enamel depends on the orientation of its ultrastructural elements.

The colour of the "brown" striae of RETZIUS depends on the increased scattering of blue light (i.e. the shorter visible wavelengths) in these regions. This is considered to be related to a slower rate of deposition of the enamel in these regions; the same amount of mineral per unit volume being present as fewer, larger crystallites - separated by larger intercrystallite spaces.

11.5. The presence of iron in the pigmented surface layer of rodent incisor enamel has been confirmed by x-ray emission analysis.(BOYDE, SWITSUR and FEARNHEAD, 1961). Iron has also been found in the pigmented layer of Sorex enamel (Insectivora - BOYDE and SWITSUR, unpublished).

(With A.D.G.STEWART) We have found that dentine is "sputtered" away more rapidly than enamel under 5 KeV Argon ion bombardment, and tuft enamel more rapidly than the surrounding (normal) enamel. These differences are considered to be related to the differences in composition (degree of mineralisation) of these tissues. However, differential sputtering-erosion rates were discovered in enamel: thus the prism structure was "etched" on the bombarded surface. This result is considered to reflect the underlying differences in crystallite orientation which are responsible for the very existence of the "prisms". The tufts were found to be some 3 or 4 prisms wide in the one specimen in which they were examined.

Appended Illustrations.

Fig. 2.31.2. Electron micrograph (X 16500) of section normal to the surface of "maturing" brush-tailed possum enamel and ameloblasts. Note the presence of numerous microvilli and intercellular spaces, tonofibrillae and membrane bound granules containing a granular (or amorphous) component very similar to that which is found on the surface of maturing enamel (REITH, 1964, Proc. Enamel Symposium, London).

Fig. 2.31.3. Electron micrograph (X 33600) of section normal to the surface of maturing rat-tailed opossum enamel and ameloblasts. The image shows a very annoying artefact, viz:- "drift" in the same direction as the long axes of the crystallites, i.e. at right angles to the surface of the enamel.

Fig. 2.35. Electron micrograph (X 14050) of transverse section of human developing enamel prisms (Pattern 3).

Fig. 2.36. Electron micrograph (X 12850) of section through "maturing" brush-tailed possum (Trichosurus vulpecula) ameloblasts at the level of their nuclei, showing crenation of the nuclear outline, clumping of the nuclear chromatin and a great abundance of microvilli and intercellular spaces.

Fig. 2.37. Electron micrograph (X 12850) of transverse section of coypu rat incisor inner-enamel prisms, showing their arrangement into clearly defined longitudinal rows (Pattern 2) with well-defined inter-row sheets of parallel oriented crystallites. (i.e. the extended, branching narrower fields of more longitudinally sectioned crystallites. INCISAL to the left.

Fig. 2.38. Electron micrograph (X 26000) of oblique (from cervical to cuspal) transverse section of Rhesus monkey developing enamel prisms, showing a well marked longitudinal row arrangement with parallel oriented sheets of inter-row crystallites (Pattern 2). Hole in support film at bottom centre. CUSPAL to the top.

Fig. 2.39. Electron micrograph (X 12850) of transverse section of developing manatee enamel prisms close to the developing front. The depressions in the mineralising front of manatee enamel are equivalent to (Pattern 1) prisms; they are not completely filled-in at the level shown. It is not certain that the rather patchy distribution of "enamel" within the filling-in prisms in this case is entirely due to the disruption of the tissue (which has undoubtedly occurred to some extent) during the polymerisation of the methacrylate embedding medium.

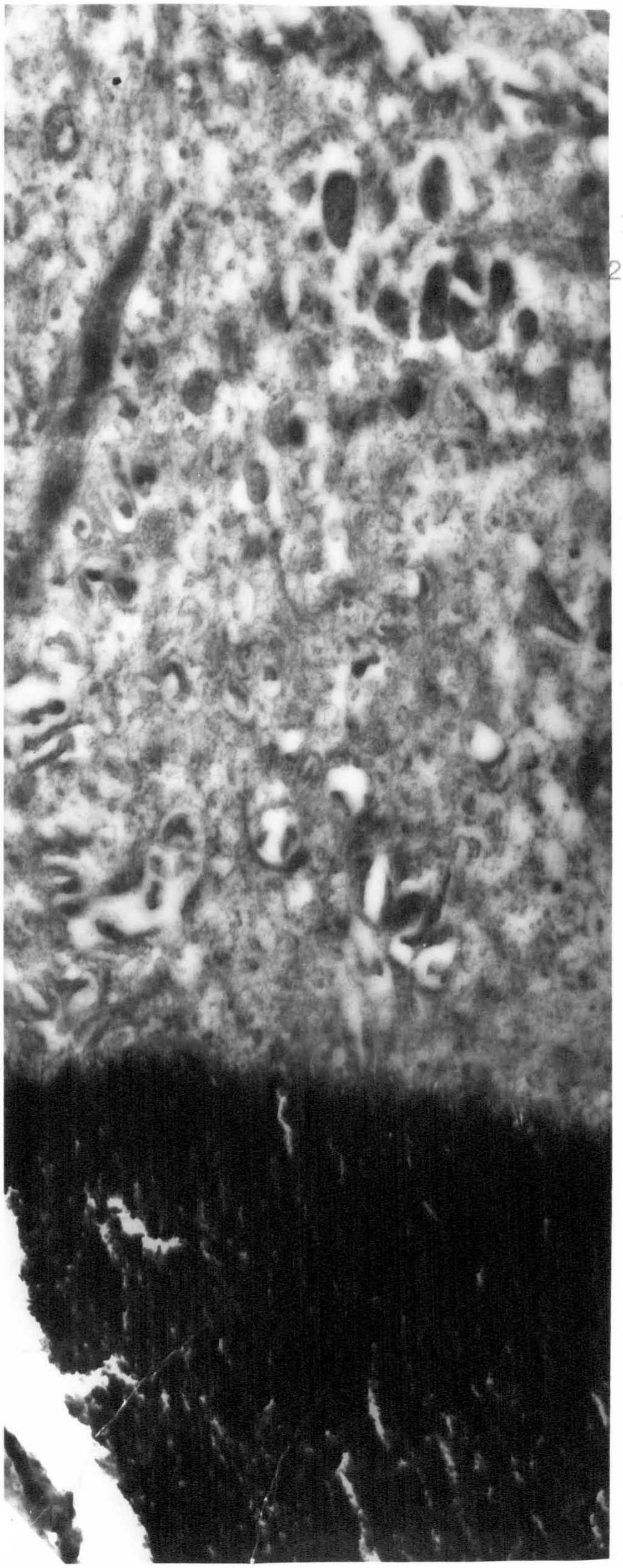
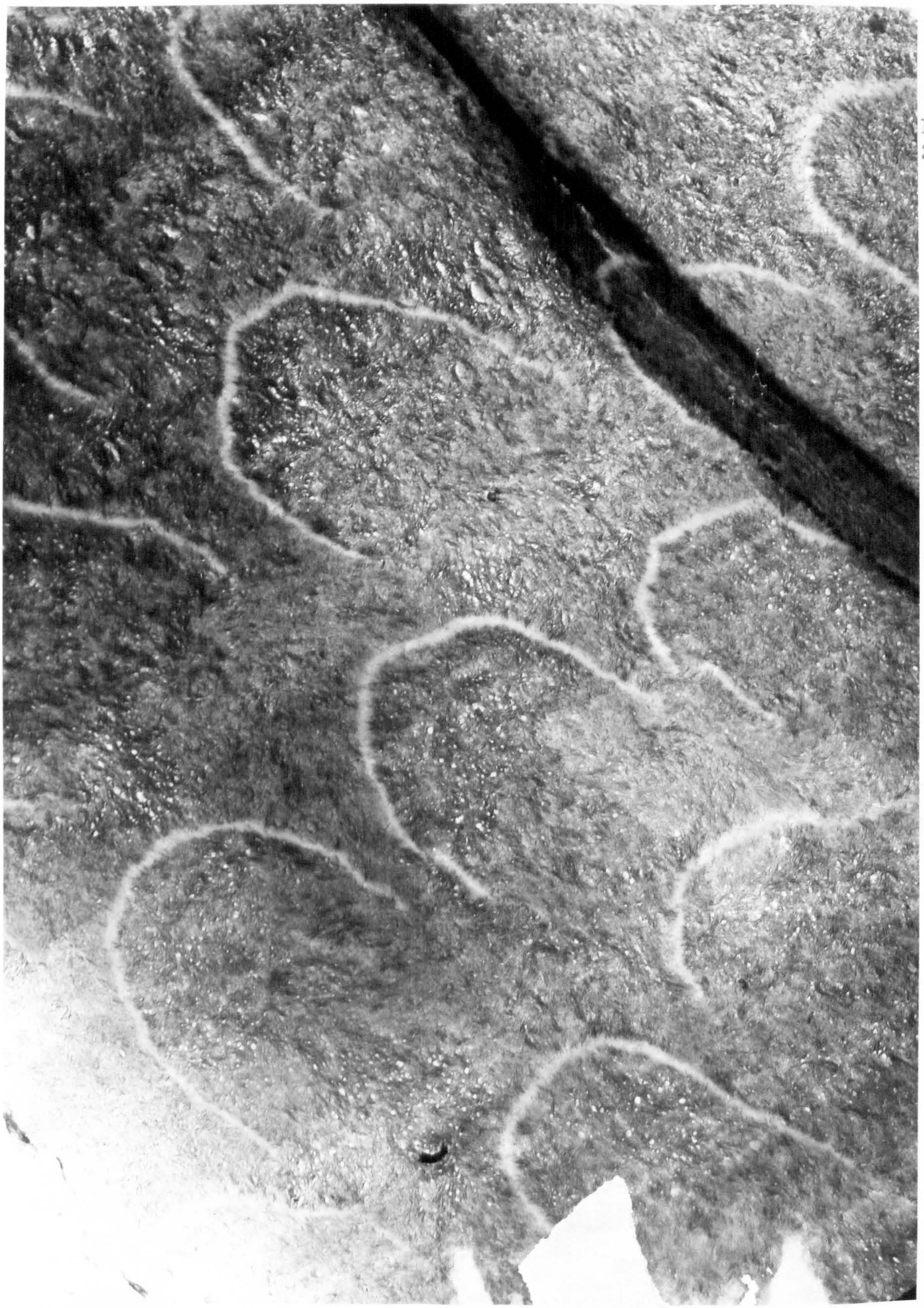


Fig. 2.31.2

Figure 2.31.2. Trichosurus vulpecula: Surface of maturing enamel and ameloblasts. (X 16500).

Fig.
2.31.2.

Figure 2.31.3. Didelphis nudicaudata: enamel surface and ameloblasts. Image drift in the same direction as the long axes of the crystallites in the enamel, viz: perpendicular to the surface of the enamel. (X 33600).



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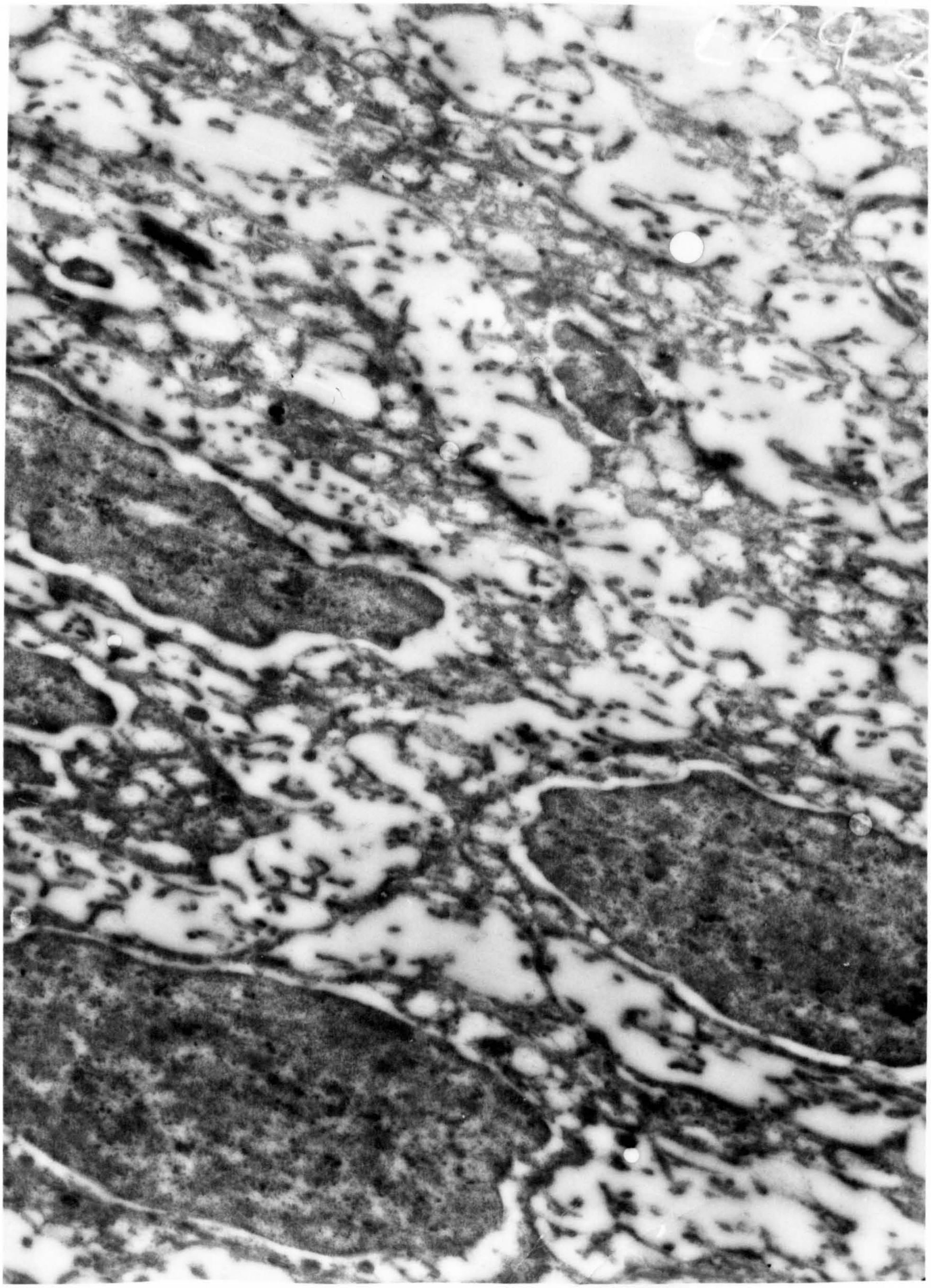
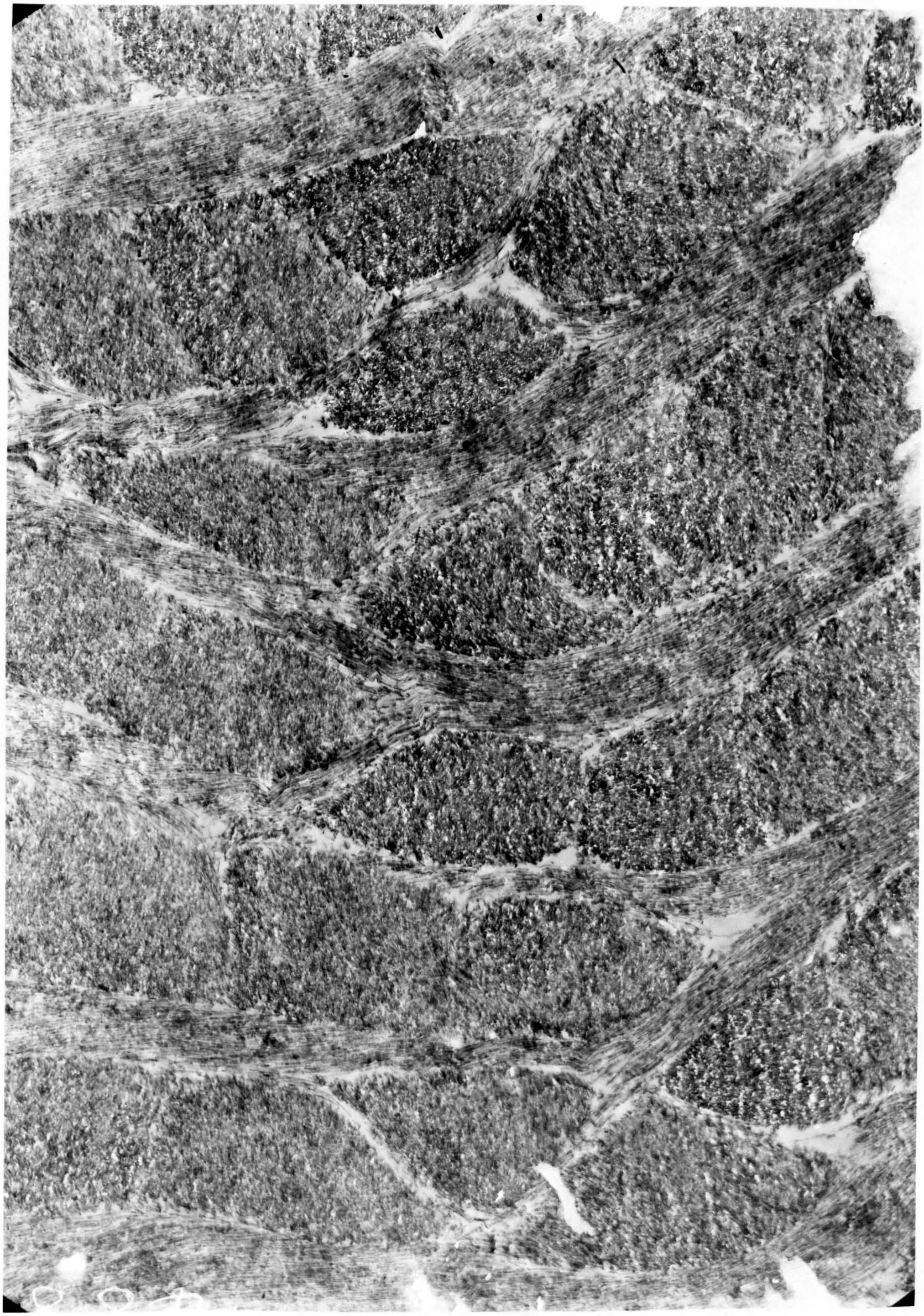


Fig. 2.36

Figure 2.36. Trichosurus vulpecula: maturing ameloblasts. (X 12850)



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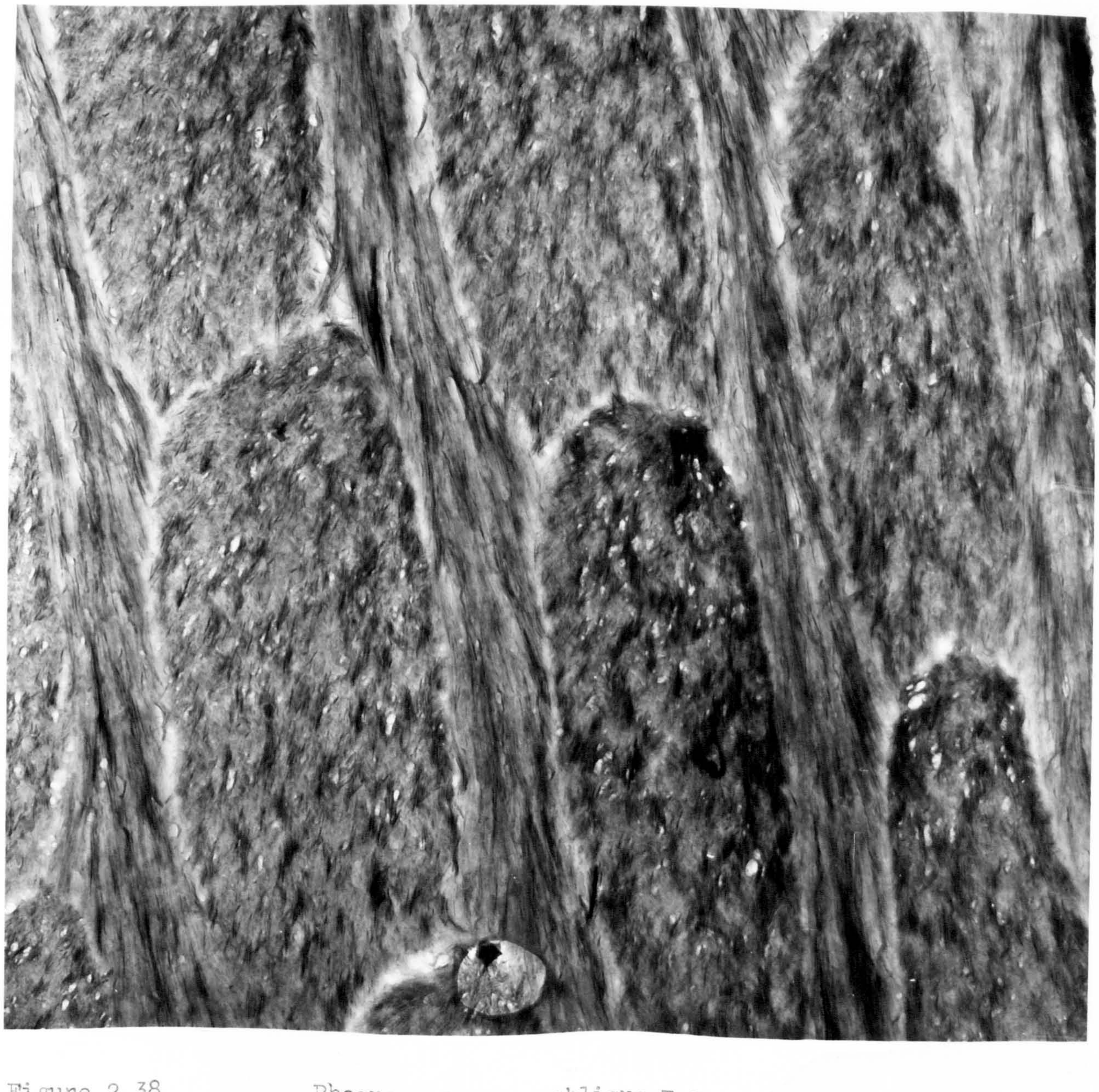

Figure 2.38 Rhesus macacus : oblique T.S. prisms. (X 26000)

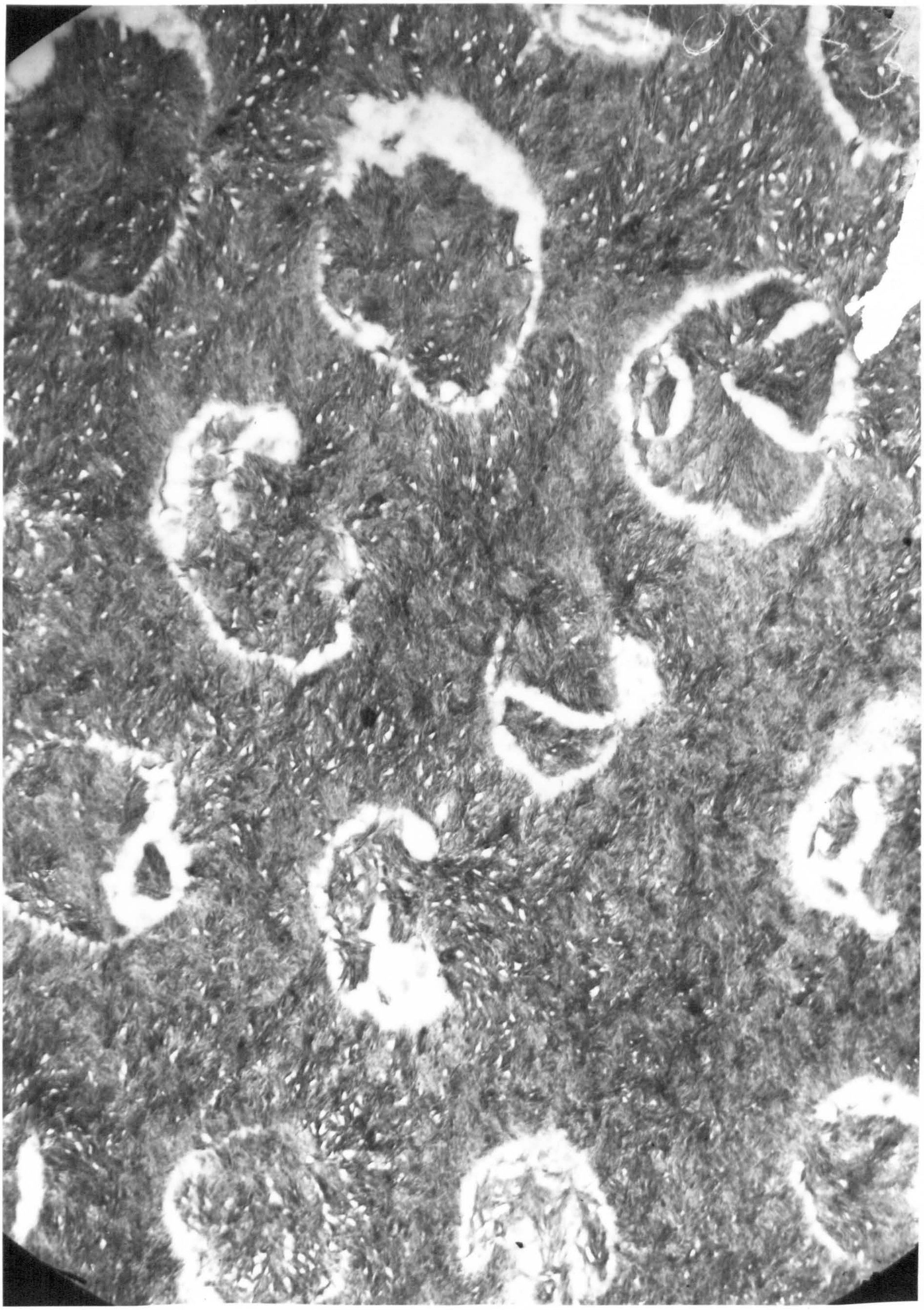

Figure 2.39 Trichecus latirostris. T.S. prisms. (X 12850)

Appended illustrations (contd.)

Fig. 2.40. Low power survey electron micrograph (X 3400) of longitudinal section of rat-tailed opossum tooth germ, showing from (top) left to right: predentine; dentine; enamel and ameloblasts. A number of the enlargements of the "enamel tubules" just inside the enamel-dentine junction can be seen (these were first described by Tomes (1849)), and at least four "tubules" which are within prism domains. One tubule crosses the enamel-dentine junction about 5 cms above the lower border of the photograph. CUSPAL to the top.

Fig.2.41. Low power survey electron micrograph (X 4050) of "Honeycomb" section (T.S. prisms) of the developing front of the enamel in a rat-tailed opossum tooth germ. This section is one of a series of 30 which were photographed in order to establish that the pattern of defects identified as "enamel tubules" was continuous through the series. The tubules are very small white specks (ca. $\frac{1}{2}$ - 1 mm. in diameter) in this micrograph: a few of them have been ringed in order to make it easier to see them. They are regions in which no crystallites have grown. The section enters the enamel surface obliquely from cervical to cuspal. CUSPAL to the left.

Fig. 2.42. Higher power electron micrograph (X 14050) of similar material - one of a series of 40 sections photographed at the same magnification. The image is marred by knife marks on the section and by heavy osmium fixative deposits at the surface of the developing enamel. Two regions identified as "enamel tubules" because they were present in a series of sections are labelled t,t. CUSPAL to the top.

Fig. 2.43. Low power survey electron micrograph (X 4050) of "honeycomb" section of developing pig permanent molar enamel. The plane of section is nearly the same as that assumed in Diagram Fig. 2.13.5: it cuts the surface of the developing enamel nearly at a tangent, entering it from CUSPAL (top) to cervical. Note the Pattern 2 prism arrangement with the early development of the inter-row sheets; the presence of some α-cytoplasmic membranes in the TCMFS' processes, i.e. in the ameloblasts below the level of the inner terminal bar apparatus - which is also prominent here. The parallel, preferred orientation of the α-cytomembrane array in the ameloblasts is in the same direction as the rows of prisms in the "enamel".

Fig. 2.44. Electron micrograph (X 20800) of section of developing rat incisor inner-enamel. The section cuts the long axis of the tooth transversely, the surface of the tooth at 45°; so that it is nearly parallel with the prism direction and also with the direction of the transverse rows of prisms. INCISAL = NW. The section passes obliquely from the filling in prisms of one row to those of the next. The prism domain crystallites are sectioned nearly longitudinally,,whereas the "interprismatic" crystallites are sectioned more nearly transversely.

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Figure 2.45. Electron micrograph (X 12600) of "honeycomb" section of developing rat incisor inner-enamel. The section cuts the surface of the developing enamel very nearly at a tangent with respect to the longitudinal direction of the tooth, but enters it obliquely from right to left (of the present field). The depressions in the mineralising front (prisms) of one set of alternate transverse rows are thus sectioned more obliquely than their neighbours. The crystallites in the more nearly transversely sectioned prisms are also sectioned more nearly transversely. Note the continuity of the "interprismatic" crystallites in the longitudinal (NS) direction; they form longitudinal sheets, but these would-be "sheets" are interrupted by the decussation of the alternate transverse rows of prisms. INCISAL to the top.

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Fig. 2.40

Figure 2.40 Didelphis: L.S. EDJ with "tubules" and their dilatations.
(X 3400)

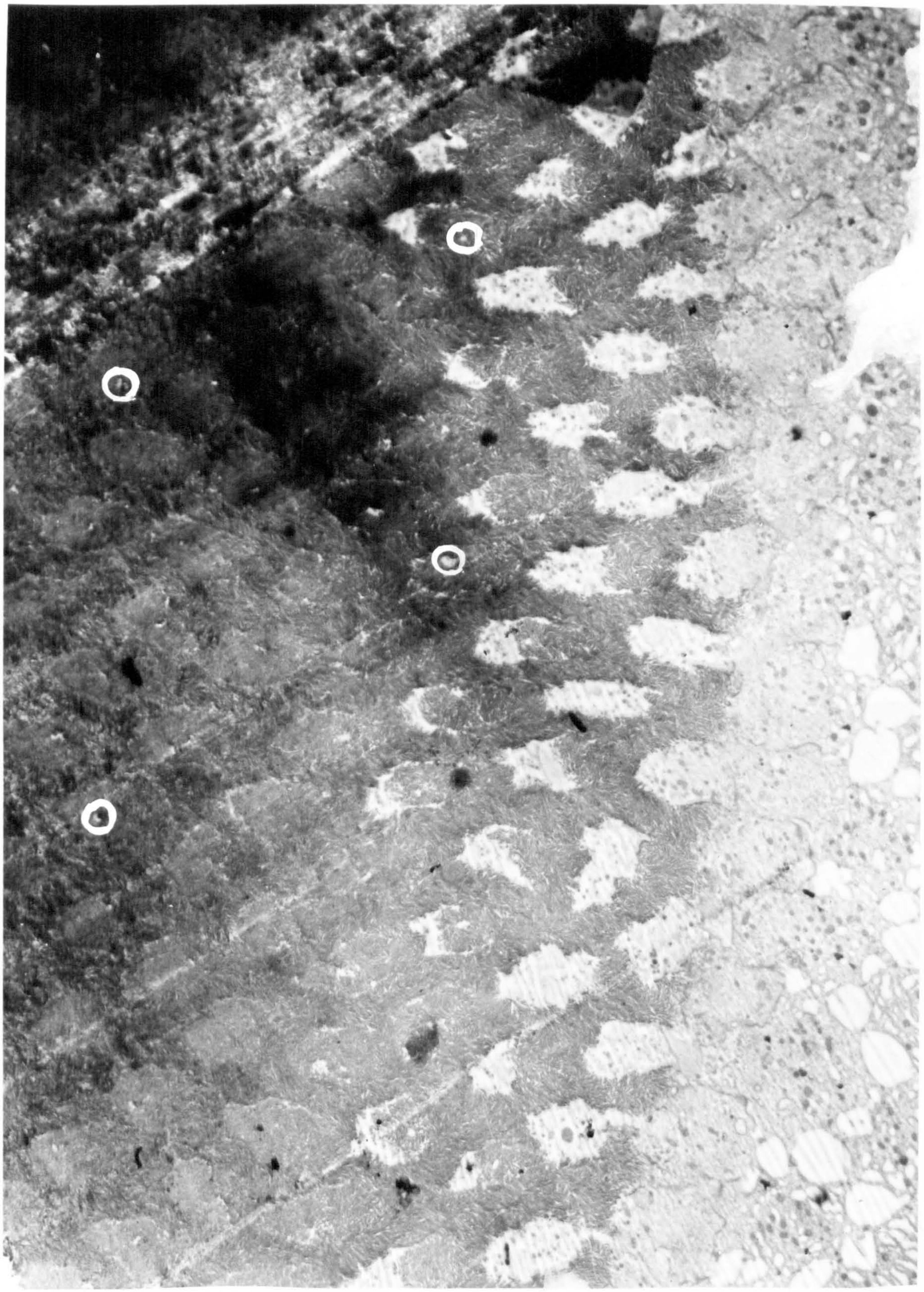

Figure 2.41 Didelphis: "Honeycomb", T.[illegible]isms w[illegible]h "tu[illegible]". (X 4050

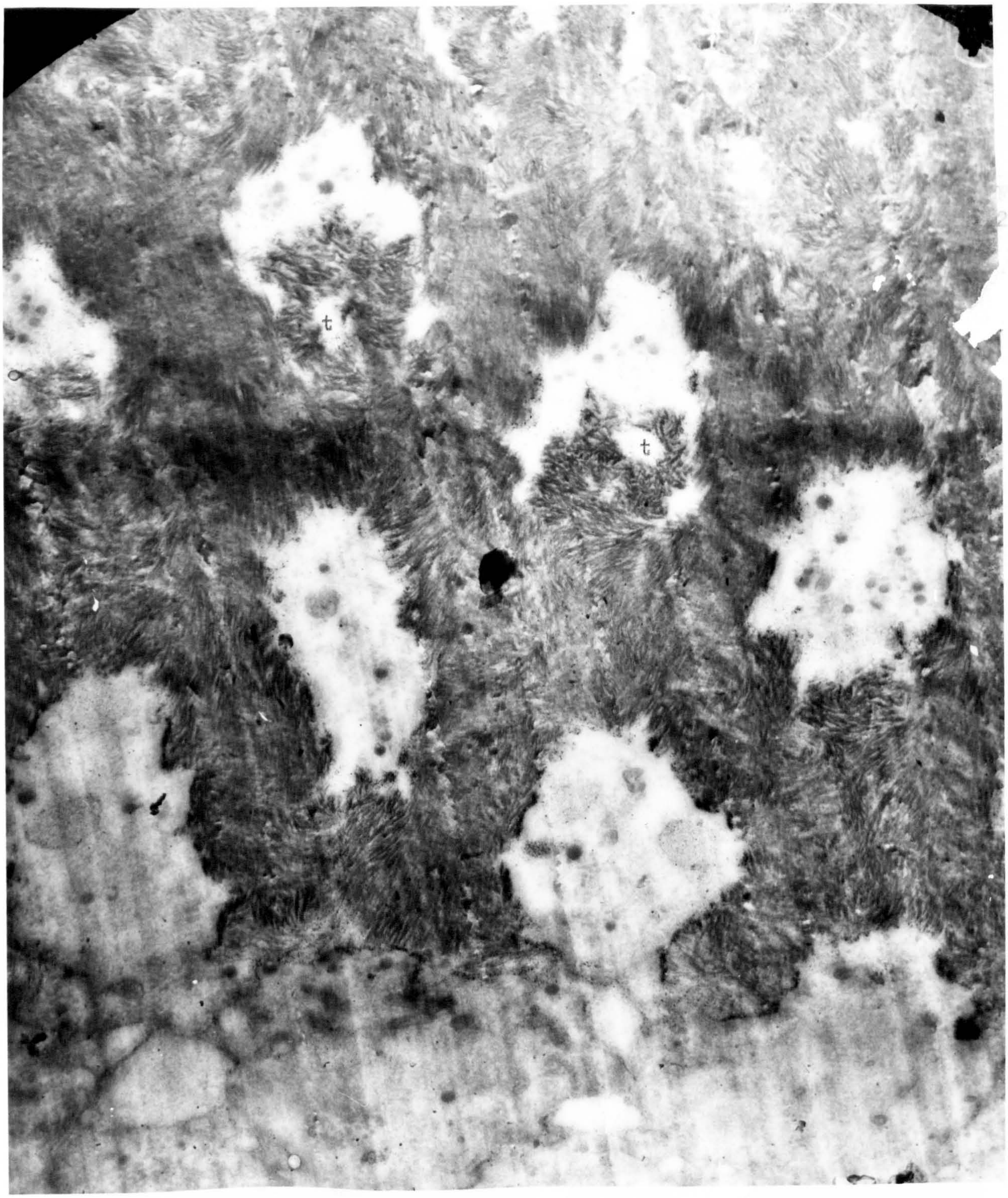

Figure 2.42 Didelphis: "Honeycomb", T.S. prisms with tubules. (X 14050)

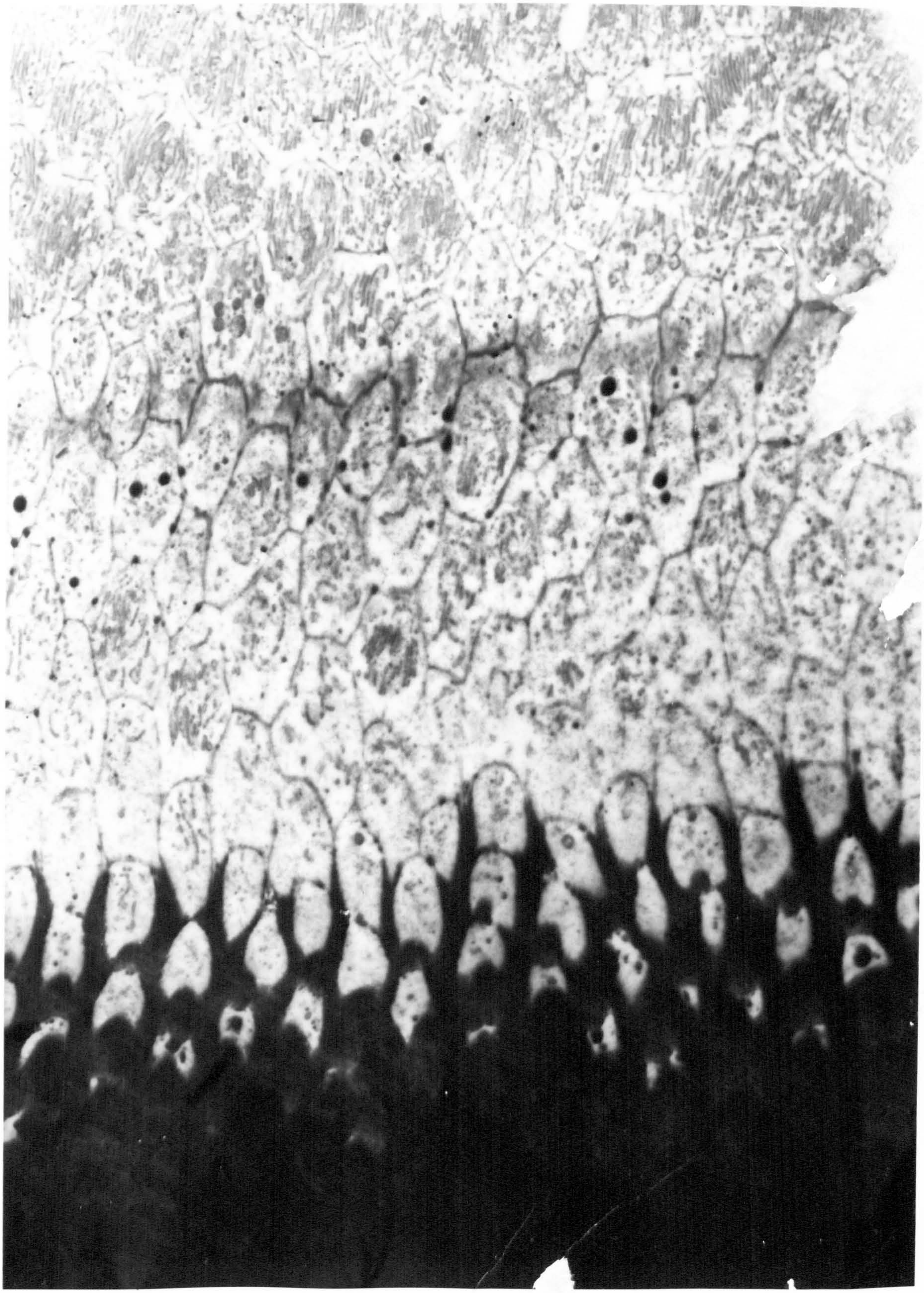

Figure 2.43 Sus: "Honeycomb". (x 950)

Figure 2.44 Rattus: L.S. prisms

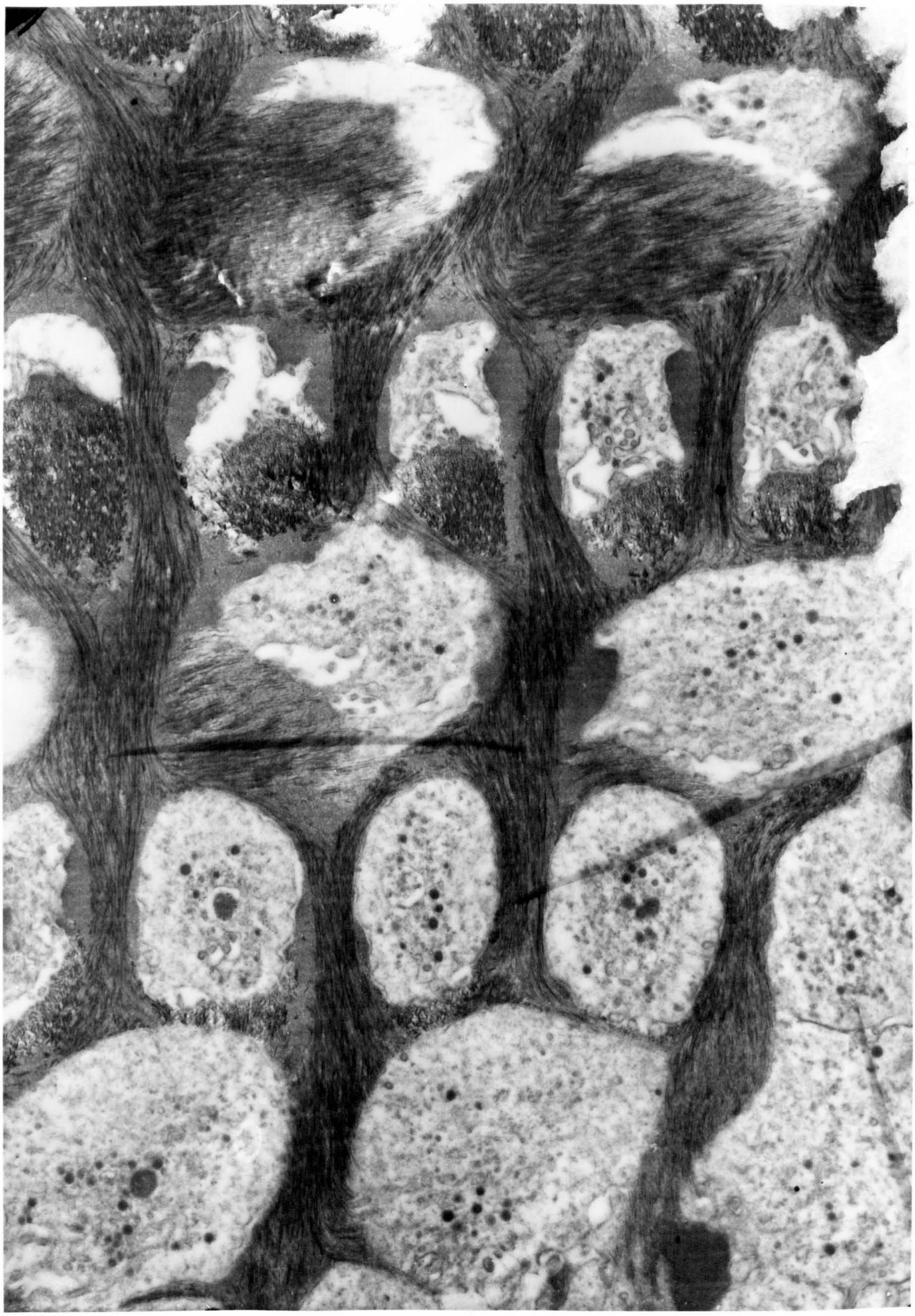

Figure 2.45. Rattus: T.S. prisms of alternate rows. (X 12600)

(Appendix A) ACKNOWLEDGEMENTS

This work has been carried out under the supervision of Professor R.J.Harrison, to whom I am greatly indebted for the opportunity he created for me and for the encouragement and facilities that he has provided: these have enabled me to undertake the studies I have reported here.

It also gives me great pleasure to make a formal note of thanks to Mr. Ronald Fearnhead, whose infectious enthusiasm will certainly prevail to start many other men over the first difficult hurdles in "research": my thanks are also due for his untiring encouragement and support since that time.

Mr. James Elliott and Dr. M. Braden have given many of their valuable hours to discussions on various topics and I am very grateful to them as well as to Mr. Fearnhead, Dr. F.R. Johnson and Professor A.E.W. Miles in this respect.

Dr. V.E. Cosslett gave his permission for the X-ray microanalytical studies to be commenced and has given a great deal of encouragement since. Dr. Roy Switsur has suffered a good deal of inconvenience with a noble lack of protest during our initial trials of his microanalyser on our tooth specimens and has given me every help and encouragement since: every moment spent in his company has been valuable as well as pleasurable.

I would like to record my sincere thanks for the devoted and interested co-operation I have received from Mr. A.D.G. Stewart, and not only in respect of our studies of ion-etching and the scanning electron microscopy of teeth. Garry Stewart was the creator of the possibility to carry out these studies, both via his organisational acumen and in that he was largely responsible for designing and building the scanning electron microscopes on which the work was carried out (at the Engineering Laboratory, the University of Cambridge and at the Cambridge Instrument Company). My thanks are also due to Mr. M.A. Snelling as head of the department of the Cambridge Instrument Company in which the development of the Microscan and Stereoscan was carried out and to Messrs. I.W. Drummond and J. Culpin for their assistance in operating the Stereoscan.

Huw. Rosser has worked hard and with extreme care at "running" our specimens in the Cambridge Instrument Company Microscan. He has lavished considerable care and attention on that instrument and without his help

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the X-ray microanalytical studies of the calcium concentration in developing enamel could not have been begun; my especial thanks are due for this assistance.

By the great courtesy of the Firma Trüb-Täuber AG, Zürich, Switzerland I was able to see their Metioscope in operation and to examine some of the specimens which I had prepared for this instrument. I would particularly like to acknowledge the courteous treatment I received from Dr. Massini of this firm and from Mr. D. Watts of their english agents (H.Tinsley & Co. Ltd. of South Norwood, London S.E.).

Mr. J. Young and Mr. P. Richards of The London Hospital Medical College and Messrs. C.B.Ellis, H. Knight. E.W. Cowl and E. Webb of The London Hospital Research Workshops have fabricated many items, large and small, without which these studies could not have progressed and I am extremely grateful to all these gentlemen for the skill and artistry which they have excercised on my behalf. The late Charles J. Washington designed and built (to an idea of Ron Fearnhead's) another unique device which I was privileged to use extensively: this was a special specimen stage for the Siemens Elmiskop which enables the grid to be tilted through 90° whilst under examination. The excellence in function of this device is a living testimony to the genius and skill of a remarkable man.

I am grateful to many people for a variety of technical hints and tips, but I am most grateful to Mr. Algy Persson (of L.K.B., Stockholm), for he taught me how to "break" glass knives.

Financial assistance for various aspects of my work has been received from the Yarrow Research Fund and from the Advisory Medical Research Committee of The London Hospital and Medical College ; from the Central Research Fund of the University of London; and from the Medical Research Council.

My employers, The London Hospital Medical College, have been very generous with financial assistance which has enabled me to travel to enjoy the hospitality of Professor J-G. Helmcke's Department in Berlin and to attend the excellent Summer School on X-ray Microanalysis organised by Dr. V.E. Cosslett at the Cavendish Laboratory, the University of Cambridge.

Many people have been of assistance in providing me with animals or already fixed material which have been used in this study; I would like to thank my brother Carl, Dr. Burns of the Carnegie Institute, Dr. J.W.S. Harris, Dr. F.R. Johnson, Dr. Alwyn Gaunt, Dr. J.D.W. Tomlinson, Major J.J. Buxton, Mr. R.C. Adams, Dr. Bryan Radden, Prof. A.M. Horsnell, Dr. Clive I. Mohammed, Mr. John Walker, Mr. R.N. Fiennes, Miss J.A. Dawson, Mr. Joseph Backhouse and many others who helped me in this way.

I would like to thank my brother Patrick and my sister Susan for the help they have given me from time to time with translations. Other members of the clan deserve my heartfelt thanks for their useful criticism and for tolerating this volume as an excuse for so long.

Mrs. Barbara Pears performed the lion's share of the typing behind this thesis and it gives me considerable pleasure to thank her for all her hard work and her skill at interpreting my manuscript. Miss Caryl Guest has also struggled with the same problem and I thank her for typing the first few chapters. I find it hard to believe that the text could ever have been brought to some sort of completion without the many hours of hard work given up by Miss Doris Sadler in correcting, adjusting and producing the final copies from stencils and it gives me the greatest pleasure to record my gratitude to her. The faults that remain with this script are all my own.

Appendix B

Some notes on the rat-tailed opossum, Didelphis nudicaudata.

Interesting experience of the husbandry of these marsupial mammals in captivity was obtained and some incidental anatomical observations were made, which, as far as I have been able to ascertain, have not been reported previously. These are,

(1) that the larynx is everted into the nasopharynx so that an anatomically separate air pathway is formed, rather similar to the situation in the Cetacea. This is known to be the case in pouch-young marsupials but it persists in the adult Didelphis nudicaudata, and

(2) that the large canine-like teeth are of continuous growth. Active growth of dentine and cement in these teeth in the adult members of this species was discovered as a result of the administration of a TETRACYCLINE antibiotic to label the calcifying tissues (see Chapter 4).

Live animals were obtained through the agency of Regent Pet Stores Ltd., London. They were flown in from South America. (Their purchase was financed by a special grant from the Yarrow Research Fund of The London Hospital Medical College). They arrived on 17/4/61 - two females with four pouch-young each and one young male. From reports in the literature we expected to have some trouble in providing these animals with a suitable diet. However, it was soon discovered that they had a great partiality for freshly killed small rodents; and they survived (see next paragraph) and grew enormously on a diet of whole mice, rats, guinea pigs and even rabbits. There was, therefore, no problem with regard to the economics of their maintainence in a large animal house. These small rodents were eaten entire - except for the skin, tail and sometimes the teeth!

On arrival the animals were all housed in the same enclosure, but in separate nesting cages. They were kept under close supervision for the first week lest there should have been any signs of disagreement between the two families, but nothing untoward was noticed. However, on 24/4/61 it was discovered that the large mother had eaten three of the young of the small mother (these had been able to leave the pouch and clamber over her fur) and the small male - leaving, considerately enough, the cheek teeth of the young male, which have since served for the preparation of

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ground sections.

... It was then that some real significance began to be attached to the difference in size and colouration of the two mothers and their offspring and it is now considered that these are sufficient to regard the two groups as at least separate varieties of the same species. The small male (weighed approx. 500 Gms), the small mother (weight approx. 1 Kg) and her surviving daughter had a much paler overall colouring than the large mother (who weighed approx. 2 Kg) and her two sons who were allowed to survive (and who grew to weigh 3 Kg and 3.5 Kg respectively). The smaller, paler opossums also had a bright orange-yellow patch on the front of the chest which was lacking in the larger ones.

One of the pouch-young was separated from the large mother on 1/5/61. Considerable difficulty was experienced in removing this animal (female, weight 10.5 grams) from its teat, the latter extending to a great length before it pulled out of the mouth. The extreme narrowness of the oral orifice and the close apposition of teat and oral epithelia probably account for this difficulty. A second pouch-young (male, weight unfortunately not recorded) was removed on 7/5/61 when nothing like this difficulty was experienced - the oral aperture being much larger.

The two remaining male pouch-young (of the larger mother) were kept in captivity until twelve months later, when one died as a result of injuries received from his brother and the other donated his brain to the cause of science. The remaining three females died from unidentified (natural!) causes: the small mother on 4/6/61; her daughter the following April and the large mother in the following May. All these animals were used in the "vital dye" experiments recorded in Section 5.10. Attempts at breeding from these animals was unfortunately quite out of the question with the stock remaining after the cannibalism episode.

Mr. G. Ruddick (of The London Hospital, Department of Photography) prepared a simply beautiful cinematographic record of these animals, which includes some views of the young in the pouch and at a later stage of the wonderful capabilities of the prehensile tail (the two male young were quite tame for several weeks after they were separated from their mother).

aC1

(Appendix C)

Some order of magnitude calculations of light transmission at a double plane interface (between two identical media separated by a lower refractive index medium).

I am deeply indebted to Dr. M. Braden for the data reproduced in this Appendix.

The accompanying diagram shows the system under consideration. When total internal reflection (path ABD) occurs there is still a transmitted component of the incident radiation (in this case in the optically less dense medium, x). However, this component is very highly attenuated and its existence is not detected in common optical phenomena. Indeed, in elementary optics its existence is never referred to. If however the medium x is of very thin section, it is possible that radiation of significant intensity may emerge (path ABC). To estimate the thickness of section of x which will permit a significant fraction of the incident radiation to emerge, electro-magnetic wave theory is used.

For monochromatic planar radiation the amplitude of the transmitted electric vector (E_t) at at depth z in the medium (x) is given by STRATTON (1941: Electro-magnetic Theory, pp. 497, 498, McGraw-Hill Book Co. Inc. - New York) as

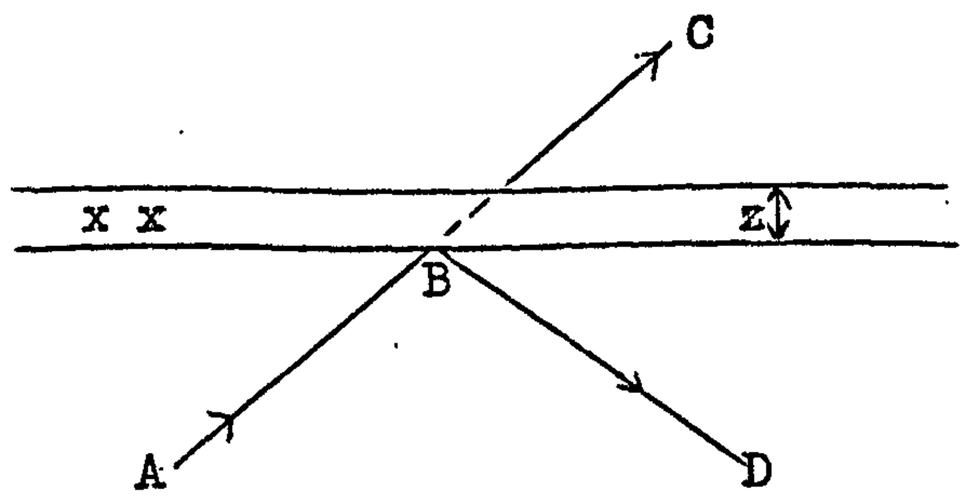
$$\frac{E_t}{E_i} = e^{-\beta_1 x} = f \quad \ldots\ldots\ldots\ldots\ldots\ldots(1)$$

where E_i is the incident amplitude and

$$\beta = \omega\sqrt{\epsilon_2 \mu_2}\sqrt{\sin^2\theta_o - n_{21}^2} \quad \ldots\ldots(2)$$

All parameters are in rationalised M.K.S. units.

$\omega = \frac{2\pi c}{\lambda}$, where c = velocity of light, and λ = wavelength.



aC2

Now for glancing incidence Sin $\theta_o \simeq 1$.

Since $n_{21} < 1$, $n_{21}^2 \ll 1$, and any variation of n_{21} with wavelength can be ignored. To a first approximation, therefore, we can neglect n_{21}^2 as being much less than unity.

Using values from STRATTON for the dielectric constant for air $\epsilon_2 = \epsilon_o = 8.854 \times 10^{-12}$ and for its magnetic permeability $\mu_o = 1.257 \times 10^{-6}$, using these values:

$\beta = 1.57 \times 10^7 \text{metres}^{-1}$ ($\lambda = 4000$Å, i.e. violet light)

$= 0.90 \times 10^7 \text{metres}^{-1}$ ($\lambda = 7000$Å, i.e. red light).

TABLE 1 of f values ($f = \frac{E_t}{E_i}$ (ratio of electric vector amplitudes))

	z = 50Å	100Å	200Å	400Å
λ = 4000Å	0.923	0.852	0.730	0.538
= 7000Å	0.955	0.914	0.835	0.700

But Intensity (i.e. Energy) is proportional to E^2.

TABLE 2 $\frac{I_t}{I_i}$ (Intensity ratios)

	z = 50Å	100Å	200Å	400Å
λ = 4000Å	0.85	0.73	0.53	0.29
= 7000Å	0.91	0.84	0.70	0.49

Conclusion

The above values show that wavelength selective reflection at a double plane interface, e.g. at an "enamel" - space - "enamel" interface, could play a significant role in determining the transmitted colour from a polychromatic source when the incident radiation has passed through a specimen in which a number of such events might occur (see also Section 8.1).

APPENDIX D

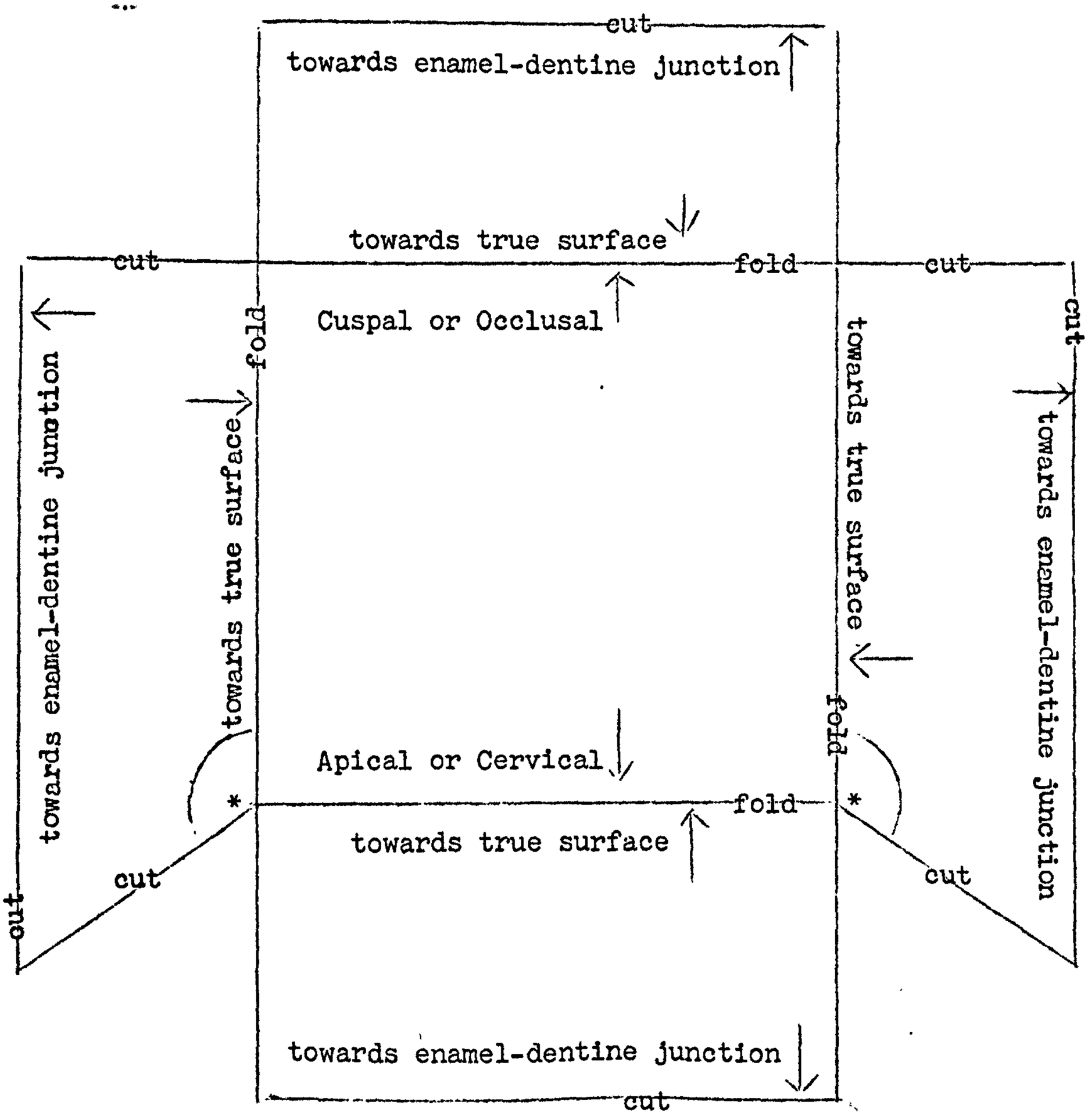


DIAGRAM Illustrating the convention adopted for representing a solid block of enamel. The angle * is the angle which the prism direction makes with the true surface of the enamel(or the surface of the developing enamel: this will be specified where it is not obvious from the context of the diagram).

b.1.

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Ion Etching of Dental Tissues in a Scanning Electron Microscope

TOOTH sections have been bombarded with a 5-kV argon ion beam inside the specimen chamber of a scanning electron microscope. This apparatus was designed so that the ion beam could be focused into a 1·5-mm diameter spot on the surface of the specimen, covering the area viewed by the microscope. The ions were extracted from a radio-frequency ion source and were focused by two electrostatic lenses on to the specimen, through two apertures. The pressure in the specimen chamber when the ion source was running was better than 10^{-6} mm mercury. The ion beam density was not uniform over its cross-section, but varied from about 10 m. amp/cm^2 at the centre to zero at the edge; this enabled us to check that, for the current densities we utilized, the nature of the changes in the specimen surface that was produced was not a function of the rate of etching. The bombarded area was kept to a minimum to reduce the amount of material deposited on the electron collection system, and reduce the temperature rise, which we have calculated to be less than 100° C, using the values of thermal conductivity given by Craig and Peyton[1]. The total ion current was kept to about 15μ amp. The micrographs were taken with a 16-kV scanning electron beam and only the high-energy reflected electrons were used to form the picture. This allowed micrographs to be taken with a resolution better than 500 Å., even though the specimen tended to charge under the electron beam. This method of observation also allows pictures to be taken while the surface is under bombardment; but for this work it was found preferable alternately to bombard for, say, 5 min, and then observe the surface at leisure. The ion beam made an angle of about 40° to the specimen normal: the direction is indicated by the arrow on the micrographs. The surface was also viewed at an oblique angle, as is indicated by the magnification symbols on the micrographs (Figs. 1 and 2).

The specimens, which were sections of teeth (human, rodent (*Myocaster coypu*) and marsupial (*Macropus*)), were polished down to a thickness of about 100μ before mounting. They were further polished on metallurgical polishing papers (down to a grade 4/0, John Oakey and Sons, Wellington Mills,

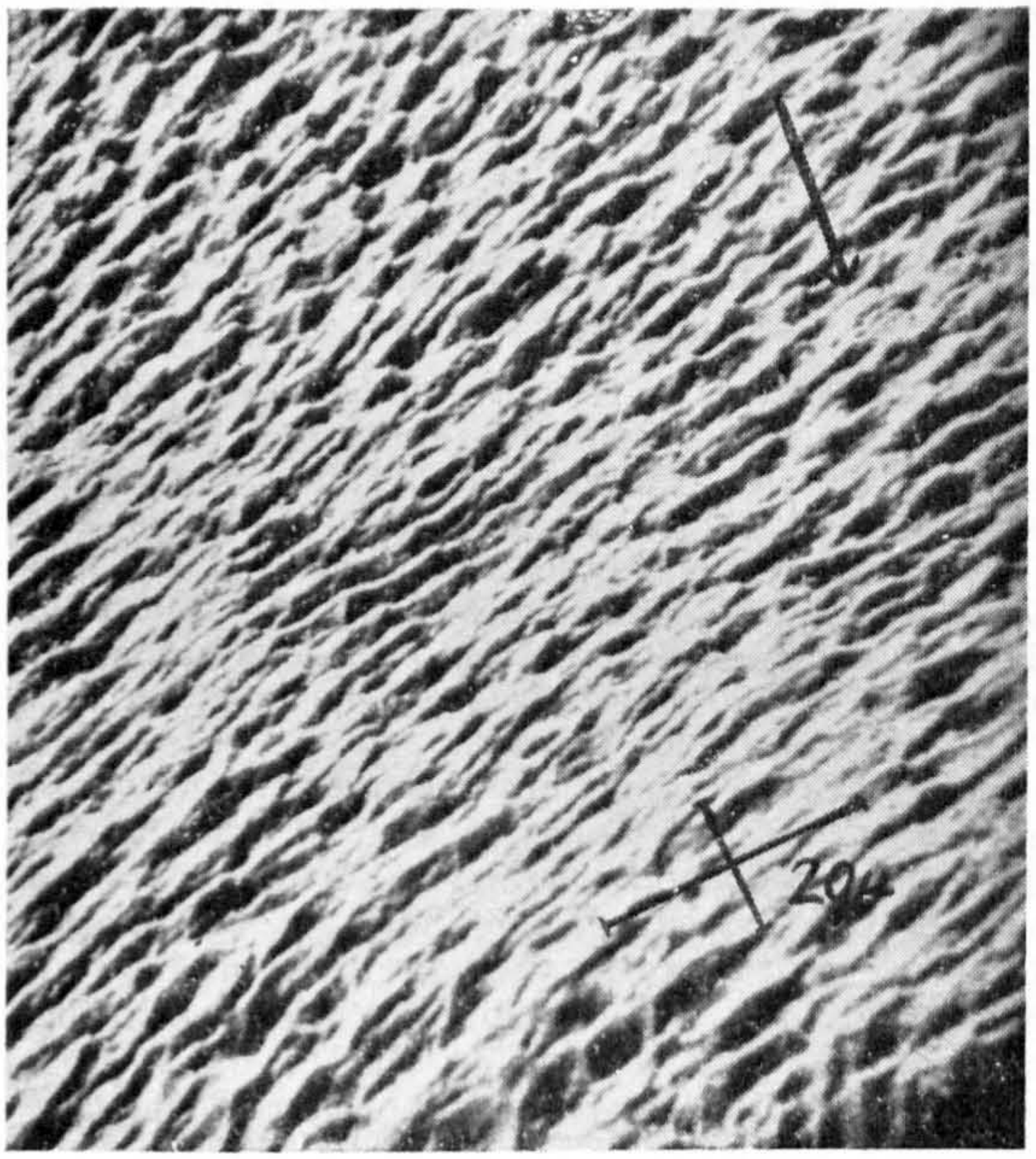

Fig. 1. Etched surface of section of *Macropus* enamel, showing enamel rods running nearly parallel to the surface

Fig. 2. Etched surface of the section of *Macropus* dentine. This region is close to the pulp chamber, hence the dentinal tubules are close together. The peritubular dentine can be seen 'standing proud' of the intertubular dentine

London, England), after sticking them with 'Araldite' adhesive to an aluminium backing plate. Before bombardment, the specimens were covered with a 300 Å. layer of aluminium to keep the electrical resistance from the specimen to earth as low as possible; this thickness of aluminium would be removed by the ion beam in less than 10 sec.

After observation in the scanning microscope the specimens were also examined by optical methods and by taking replicas, which were studied in a Siemens electron microscope. The surface was found to be hard, and true replicas could be produced without pulling crystallite away from the surface.

For a given ion beam, the rate of etching depends on the chemical composition of the specimen at the surface and on the angles that the ion beam and the crystal lattice make with the specimen surface.

In a region of constant composition, the production of a surface topography due to differential etching-rates can only depend on differences in the orientation of crystalline structural units that have been etched away. Enamel has a fairly uniform composition, but it was etched differentially to produce a surface profile in which the rod structure of the tissue could be visualized (Fig. 1). We suggest, therefore, that the differences in sputtering-rate which we have observed in enamel are related to the differences known to exist in the orientation of the crystallites of hydroxyapatite[2], and that ion beam etching may have a potential as a method for revealing these differences. Dentine was etched away more rapidly than enamel: enamel is known to be the more highly mineralized. Intertubular dentine was eroded more rapidly than peritubular dentine (Fig. 2): the latter is again known to be the more highly mineralized. We suggest that these differences in sputtering-rates are primarily related to these differences in mineralization.

The scanning electron microscope proved of great value in enabling the etching to be followed, until a convenient amount of material had been removed. As the specimen was bombarded in its correct position for viewing by the scanning electron microscope, previously observed areas could be rapidly re-located after each period of bombardment.

Although Figs. 1 and 2 are scanning electron micrographs, replicas were taken of the specimen surface after it had been removed from the scanning microscope, and these were examined in a transmission electron microscope (Siemens Elmiskop I).

Good replicas were obtained, and there was no sign of any material having been pulled away from the surface with the replica, which is a common fault when replicas are taken of surfaces that have been etched with chemical solutions.

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[1] Craig, R. C., and Peyton, F. A., *J. Dent. Res.*, **40**, 411 (1961).
[2] Helmcke, J. G., Schulz, L., and Scott, D. B., *J. Dent. Res.*, **40**, 668 (abst.) (1961); and personal communication from J. G. Helmcke.

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Scanning Electron Microscopy of the Surface of Developing Mammalian Dental Enamel

THE surface of the developing dental enamel of the manatee (*Trichecus latirostris*) and the monkey (*Rhesus macacus*) has been examined directly in a scanning electron microscope. Molar tooth germs were dissected from the jaws of these animals soon after death. Specimens were prepared by gently stripping off the enamel organ from the surface of the developing tooth, thus exposing the developing front of the enamel. The pulp was then removed from the centre of the developing tooth, which was divided into convenient portions and allowed to dry. These portions were fixed with 'Araldite' adhesive to aluminium specimen stubs leaving the enamel side exposed. The specimens were finally covered with a 300-Å thick layer of aluminium to render their surfaces conducting. They were examined in a scanning electron microscope (a developmental model built by the Cambridge Instrument Co., Cambridge, England). The micrographs were taken with a 15-kV scanning electron beam and only

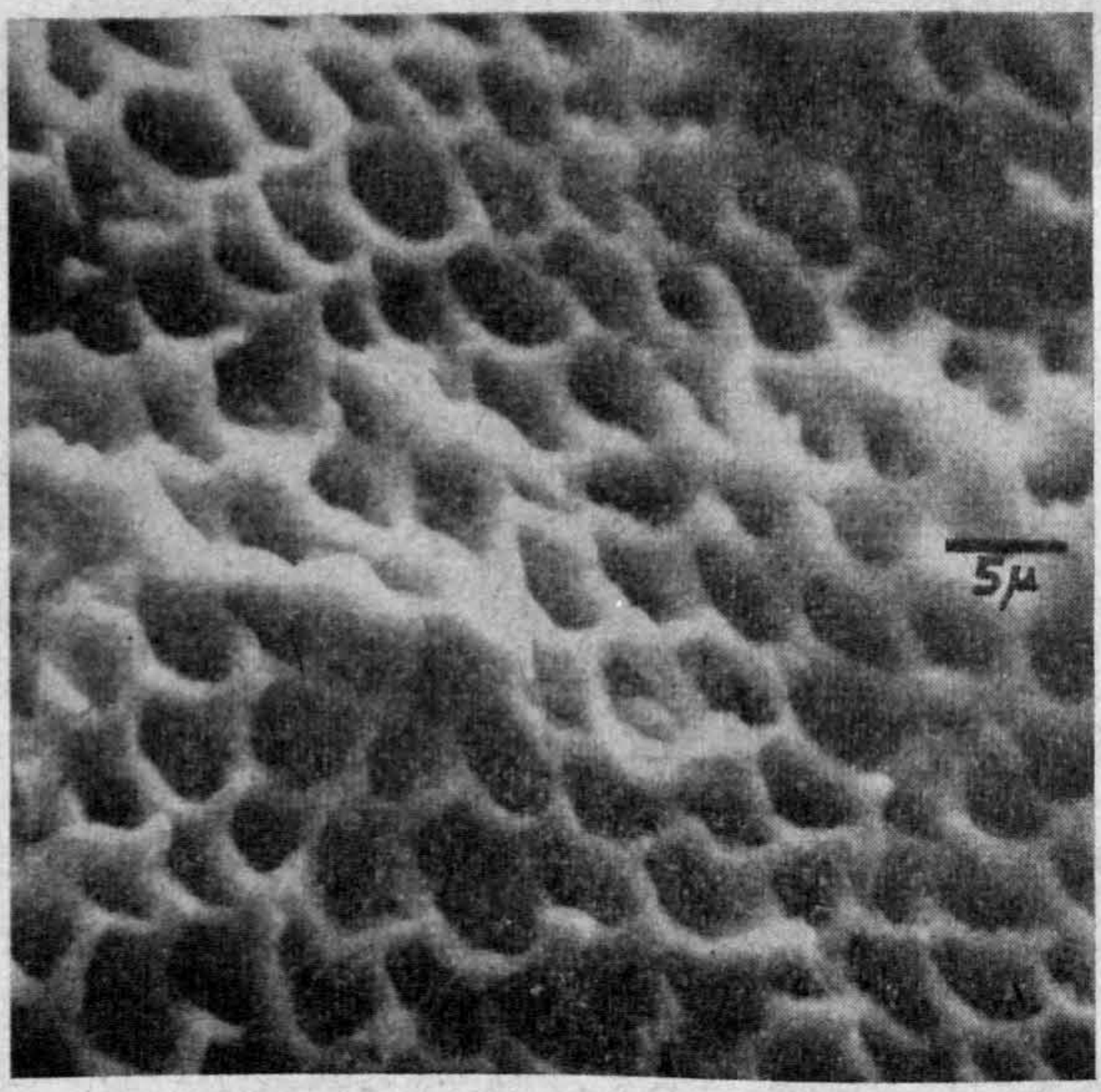

Fig. 1. Scanning electron micrograph of developing surface of manatee enamel, showing depressions which had been occupied by the Tomes's processes of the ameloblasts

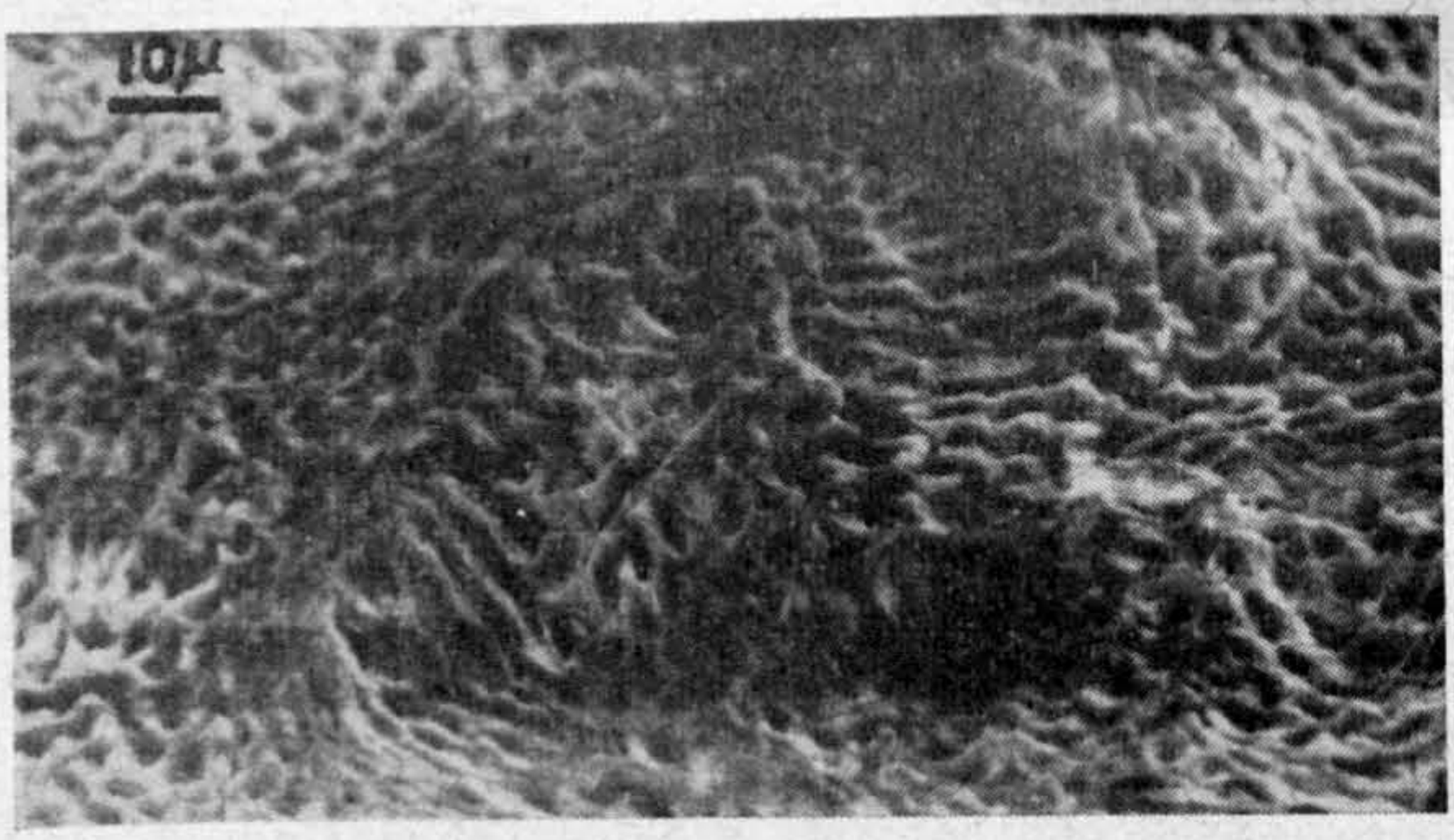

Fig. 2. Scanning electron micrograph of developing surface of rhesus monkey enamel

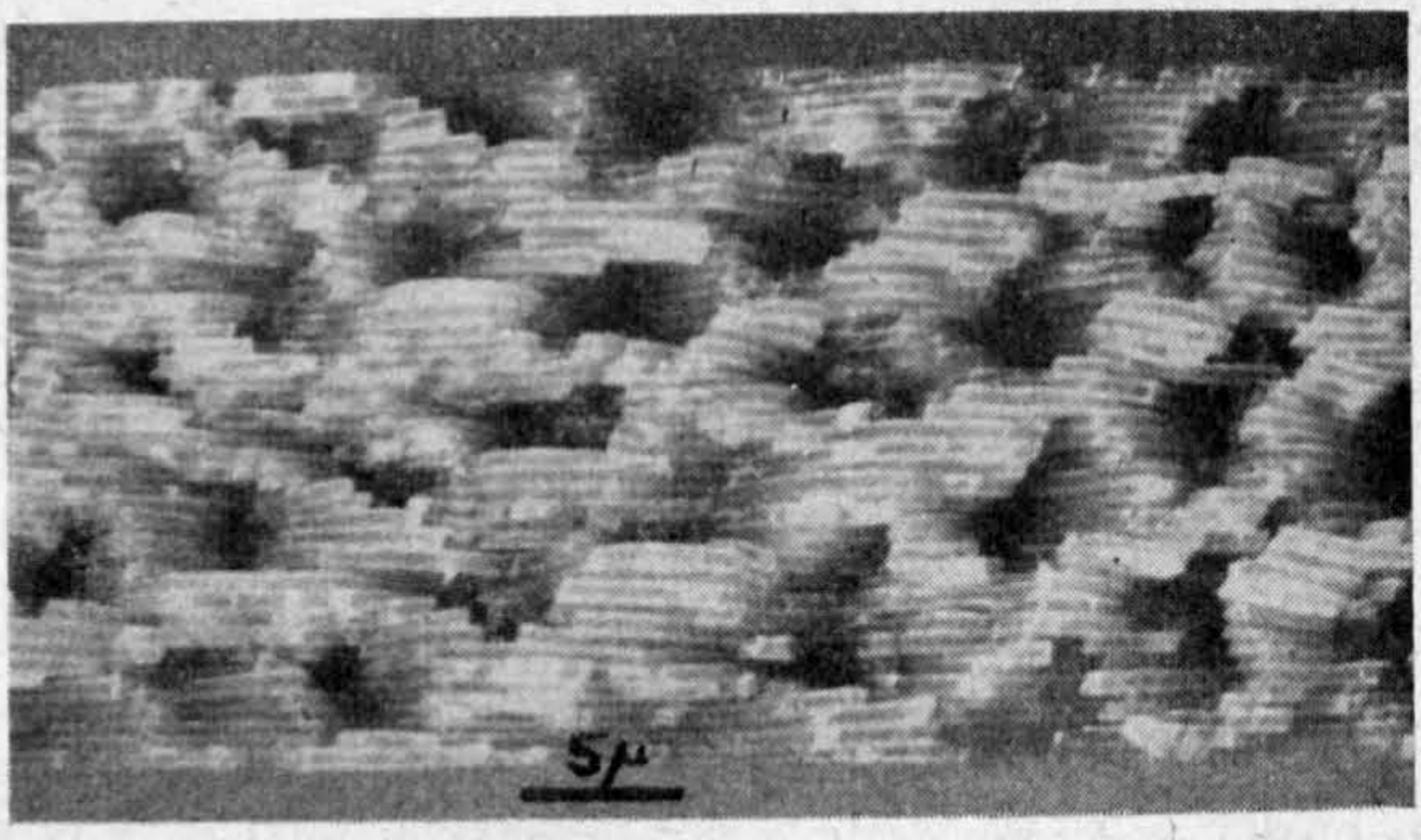

Fig. 3. Photograph of part of a wax reconstruction of the surface of developing rhesus monkey enamel

the high-energy reflected electrons were used to form the images.

The topography of the surface of the developing enamel can be seen in Figs. 1–3. The depressions in the surface were originally occupied by the Tomes's processes of the ameloblasts. In the manatee (Fig. 1) these depressions all face in the same direction. In the monkey (Fig. 2) there is a more complicated pattern with groups of depressions facing in different directions. It is known that the prisms in the enamel of the manatee are fairly straight and parallel with one another. In the monkey (and man) groups of prisms cross over each other in their course from the enamel-dentine junction to the surface of the enamel. In ground-sections examined with the light microscope at low magnifications, these groups of prisms can be seen as alternate light and dark 'Hunter-Schreger' bands. It is probable that the appearance described is associated with the development of this pattern of weaving of the enamel prisms.

The developing surface of the enamel was also observed to vary in height over larger intervals corresponding to several prism (depression) widths.

The appearances seen in the scanning electron micrographs independently confirmed the interpretation of the nature of the surface which had been made by one of us (A. B.) from an examination study of wax reconstructions. Serial 0·5μ sections of methacrylate embedded tooth germs were cut using a Porter-Blum ultra-microtome, stained with crystal violet and basic fuchsin and mounted in D.P.X. The outline of the developing surface of the enamel in each section was traced in projection using a light miscroscope at a magnification of some 1,500 diameters. Wax sheets were cut following the outline of these tracings. The surface of such a wax reconstruction (Fig. 3) shows a morphology similar to that seen in the scanning electron micrographs.

So far as we are aware, the foregoing features of the surface of developing enamel have not been described before, and would be difficult to study by other means, since newly secreted enamel is too soft to replicate in the fresh state and too absorbent to replicate in the dry state.

We thank the Chairman of the Cambridge Instrument Co. for permission to use the instrument and publish the micrographs and M. J. Culpin and I. W. Drummond for operating the instrument.

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A Study of the Etching of Dental Tissues with Argon Ion Beams

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Received January 20, 1961

Enamel and dentine have been etched with 5 kV argon ions in a vacuum of 10^{-6} mm Hg. The specimen surfaces were examined in the scanning electron microscope in which the etching took place, and afterwards, by replica techniques. The etched surface was ideal for replicating; it was clean and hard. True replicas were produced; no crystallites were pulled away from the surface. It is suggested that the differences in etching rates observed were related to differences in mineral content and crystallite orientation.

Metallurgists have found ion-bombardment using glow discharges a useful method for etching metal surfaces to reveal their structure, and more recently extensive work has been done with ion beams to investigate the basic mechanism of sputtering, (i.e., the removal of material resulting from ion bombardment) both from the theoretical and the experimental aspects (high energies, Almén and Bruce (*1*); low energies, Wehner and Rosenberg (*23*); dielectrics, Spivak *et al.* (*19*); theoretical, Nelson and Thompson, *12b*). The rate at which material is removed from the surface of a crystalline substance by a given ion beam depends upon the angles which the incident ion beam and the crystal lattice make with the surface, upon the crystal structure, and upon the type of constituent atom. This is true for both metallic and ionic crystals.

The orientation of the hydroxyapatite crystallites in dental tissues has been studied by means of polarized light (*15*; Lyon and Darling, *12a*) by X-ray diffraction (*9*, *14*, *21*), by replica techniques for the electron microscope (*10*, *12*), and by direct visualization and selected area electron diffraction of ultrathin sections in the electron microscope (*6–8*).

This paper describes some preliminary experiments that have been made to determine whether polished surfaces of the dental tissues would be eroded by argon ion bombardment in a way that could be related to the underlying structure. Special attention was given to possible relationships between the etching patterns produced and the crystallite orientation in enamel as determined by previous workers using the techniques mentioned above. The possible advantages of the use of a scanning electron microscope for this type of work were also investigated.

EQUIPMENT

Ion bombardment was carried out in the specimen chamber of a scanning electron microscope, with an attached ion source (Fig. 1) arranged so that it was not necessary to move the specimen or to break the vacuum between the operations of etching and viewing.

The scanning electron microscope used had three electrostatic lenses, magnetic deflection, electrostatic correction for astigmatism, and a working distance of 1 cm. The instrument was prealigned, except for the final aperture and the gun anode. The resolution obtained in this work was 400 Å. A more detailed description of the construction and operation of this apparatus is given by Stewart (*20*), and more details of the design and applications of scanning electron microscopes by Smith (*17*), Smith and Oatley (*18*), Wells (*24*), Everhart (*4*), and Thornley (*22*).

The ions were extracted from a conventional radio-frequency ion source. After leaving the source the ions were focused onto the specimen by two electrostatic lenses, through two apertures. These apertures separated two of the vacuum systems and also limited the area under bombardment. The pressure in the specimen chamber was less than 10^{-6} mm Hg, and there was no noticeable increase in this pressure when the gas supply to the ion source was turned on.

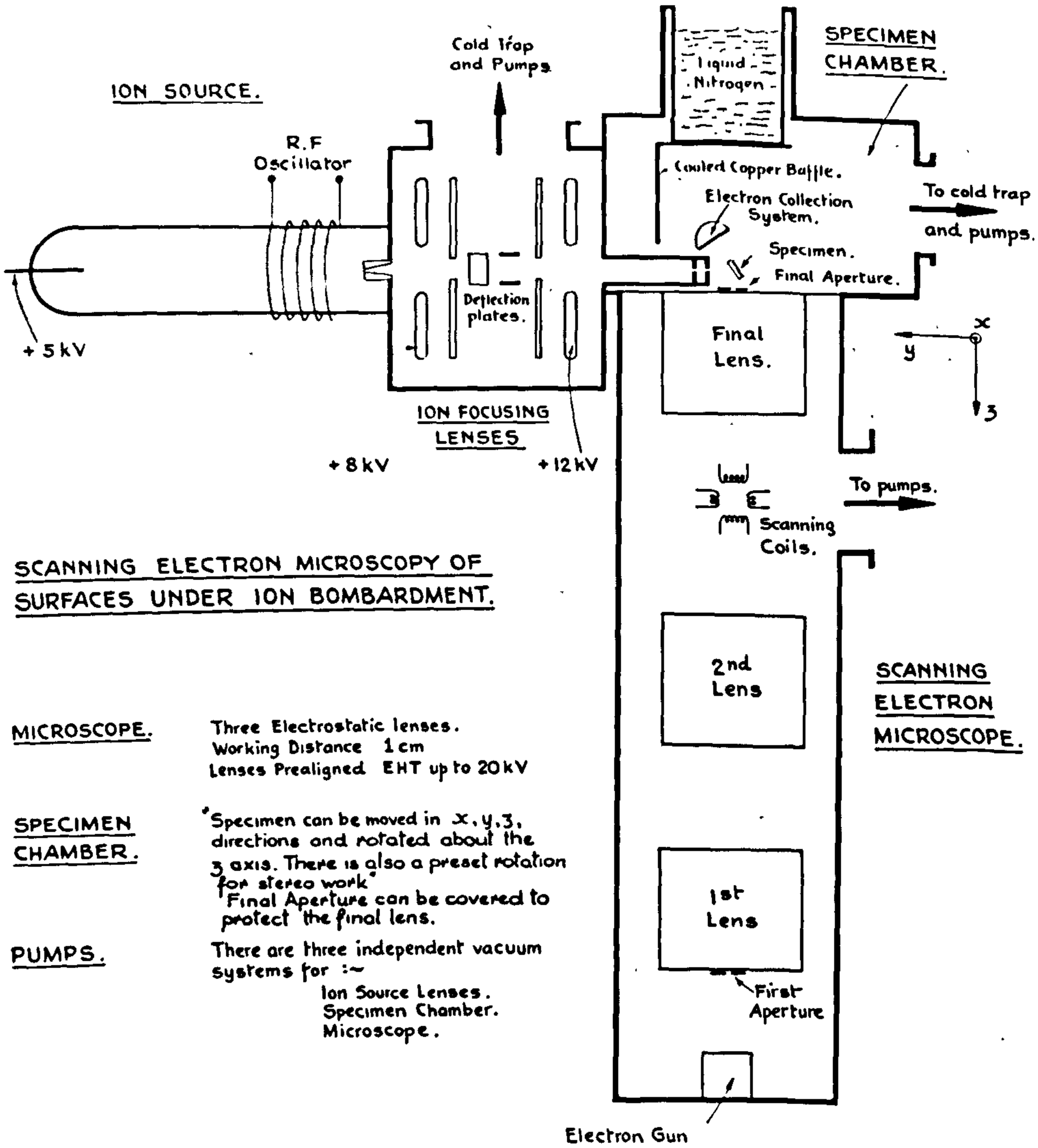
The stereo-motion of the specimen stage was preset to 8.4°.

MATERIALS AND METHODS

The specimens were sections of teeth: human, (two specimens), rodent (*Myocastor coypus* —one specimen) and marsupial (*Macropus*—one specimen). Before mounting, the sections were polished down to a thickness of approximately 120 μ and photographed with transmitted light at a magnification of ×60. The sections were then mounted by sticking them to a backing plate of aluminum or brass with Araldite or Eastman 910 Adhesive. The surface of the section was then polished on metallurgical polishing papers (down to grade 4/0—John Oakey and Sons, Wellington Mills, London, England), so that the final thickness of the section was approximately 100 μ. The finished surface was cleaned by stripping off Formvar replicas, and finally it was covered with a layer of aluminum, approximately 300 Å thick, to keep the electrical resistance between the specimen and earth as low as possible. This layer of aluminum would have been removed from the etched area within seconds of the commencement of bombardment.

The surfaces were etched with 5 kV argon ions at an angle of incidence of about 45°. An inert gas has been used both to avoid chemical effects, and because the inert gases have the highest sputtering coefficients. The factors that influenced the choice of ion beam voltage are that: there is a voltage which will give the minimum heated dissipation in the specimen per atom removed (0.2–9 kV); it is difficult to obtain a high current density with low voltage ion beams (less than 2 kV); the design of the equipment is simplified if the ions can be accelerated straight from the ion source, and for radio-frequency and for the discharge type of ion source the optimum running voltages are in the range of 3–9 kV. An accelerating voltage of 5 kV was therefore chosen for this work.

In etching the specimen from which most of the illustrations in this paper are taken, the total ion charge used was 0.09 coul, delivered in short periods over a total period of two



SCANNING ELECTRON MICROSCOPY OF SURFACES UNDER ION BOMBARDMENT.

MICROSCOPE. Three Electrostatic lenses.
Working Distance 1 cm
Lenses Prealigned EHT up to 20kV

SPECIMEN CHAMBER. Specimen can be moved in x, y, 3, directions and rotated about the 3 axis. There is also a preset rotation for stereo work.
Final Aperture can be covered to protect the final lens.

PUMPS. There are three independent vacuum systems for :~
Ion Source Lenses.
Specimen Chamber.
Microscope.

FIG. 1. Schematic diagram of equipment.

N.B.: The specimen surface was not at right-angles to the scanning electron beam and the resulting pictures (Figs. 3, 4, 5 and 6) are, therefore, foreshortened in one direction: hence, linear magnification is unequal in different directions. The two arms of the symbol × accompanying Figs. 3, 4, 5 and 6 represent the same distance in the two axes shown (which are the major and minor axes of an ellipse).

The direction of the ion beam is marked on the micrographs by an arrow. As the electron beam and the ion beam were perpendicular to each other, this arrow should be visualized as lying in the plane of the micrograph, i.e., perpendicular to the direction of view.

hours. The current density in the center of the bombarded area was about 10 mA/cm^2. The area bombarded was kept to about 1.5 mm diameter to reduce heating effects and also to limit the amount of the material deposited on the scintillator (electron collector). The total ion beam current was then between 8 and 20 μA. In these particular experiments we did not make the measurements which would have enabled us to estimate the actual ion current density on any given point of the specimen.

We have considered the possibility that the changes observed in our specimens were caused by effects other than ion etching. The possibility of a chemical attack can be excluded, since we worked with inert gas ions in a vacuum of better than 10^{-6} mm Hg. In view of the current densities employed in the ion beam, the pressure in the specimen chamber of less than 10^{-6} mm Hg, was low enough to reduce to a negligible level, the effect of hydrocarbon contamination. Thermal damage to the specimen surface is unlikely, although it was not possible to measure the temperature rise directly. As far as is known, no one has determined any values for the thermal conductivity of enamel and dentine when they have lost their moisture content under high vacuum. Therefore, the rise in temperature of the specimen surface could not be calculated precisely. But in the absence of other data we used the values of thermal conductivity for enamel and dentine derived by Craig and Peyton (3), and calculated that a temperature gradient of approximately 100°C existed across the thickness of the specimen in the area of the maximum ion beam density. This temperature rise would not be sufficient to damage the crystal structure of hydroxyapatite. In making this estimate we ignored the effects of radial cooling since the distance from any point of the surface of the specimen to the backing plate was only 100 μ.

We also assume that the only effect of the vacuum on the specimen was the loss of uncombined water. This was confirmed by examining replicas, taken after removing the specimens from the scanning microscope, of those areas which had not been bombarded: we found them similar in all respects to replicas of the original surface before exposure to the vacuum.

A study of similar areas of the specimen which had been etched to the same depth, but at different rates of erosion, showed that the profile produced was only a function of depth and was not related to the rate of removal, at least for ion current densities equal to, or less than, those we used. It was possible to study bombardment damage at different erosion rates because the distribution of ion density was not uniform, but peaked in the center of the ion beam (Fig. 2). One would only expect etching effects to be *other* than a function of depth at high etching rates, when the specimen might be overheated, or at low etching rates at room temperature, when there might be interference from hydrocarbon contamination.

In taking the scanning electron micrographs shown in this paper (Figs. 3, 4, 5 and 6), only the high-energy reflected electrons from the scanning electron beam were used to modulate the display. This allowed good micrographs to be obtained even though the surface tended to charge under the electron beam. This method of collection also allows pictures to be taken while the surface is under ion bombardment. It should be noted that the specimen surface was not at right-angles to the scanning electron beam and the resulting pictures are, therefore, foreshortened in one direction. Thus linear magnification is unequal in different directions. The axes of maximum and minimum magnification, which are at right-angles to each other, are indicated by the symbol accompanying the micrographs. The slope of the surface can be easily appreciated by studying the stereo-pairs in Fig. 3.

To determine the rate at which the specimen surface was removed, nickel particles roughly

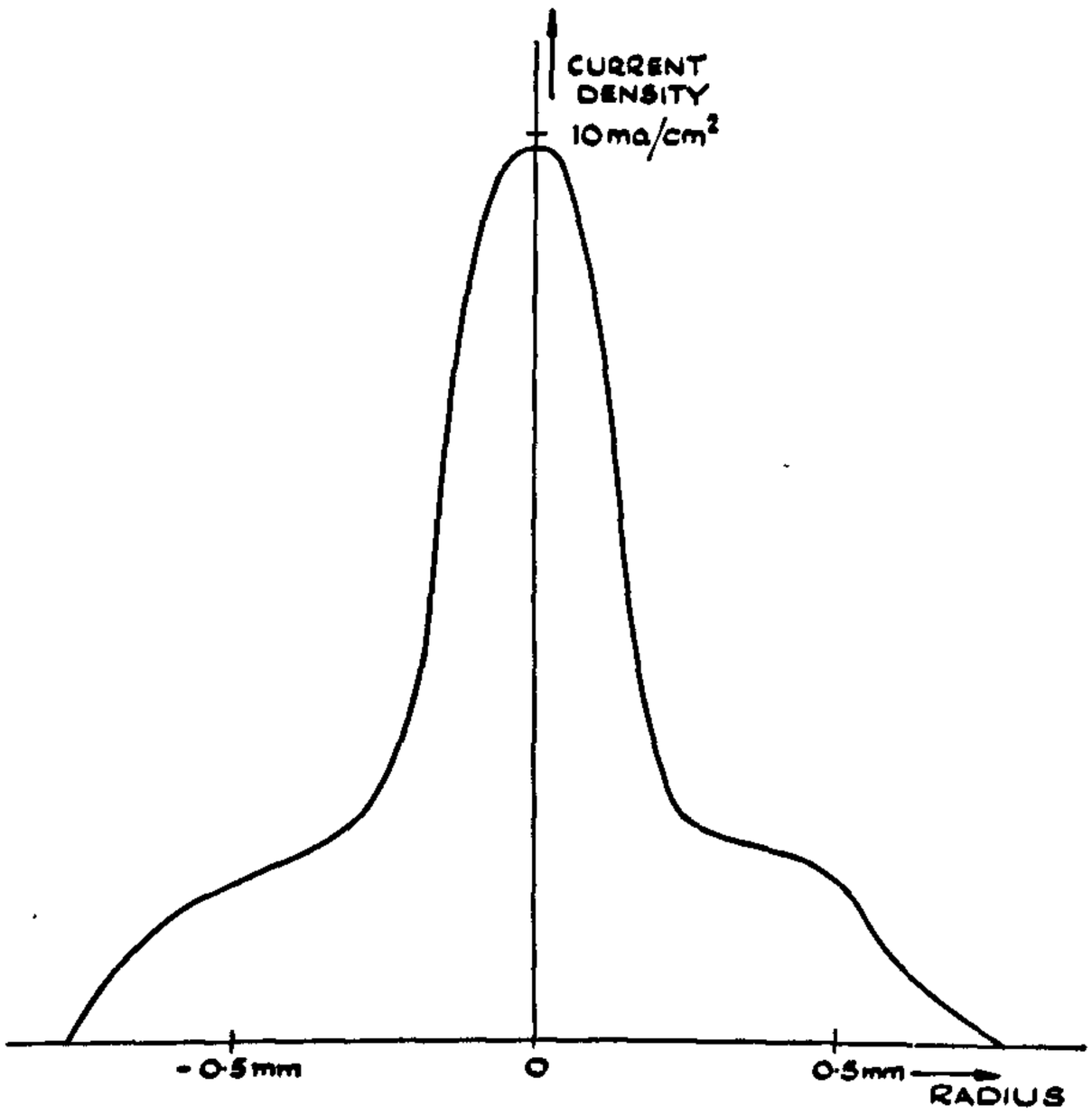


FIG. 2. Experimentally determined profile of an argon ion beam—typical of those used in this work.

5 μ in diameter were dusted onto the surface and allowed to adhere to it before placing it in the instrument. Thus, when bombarded, the original surface level was preserved under the shielding nickel particle while the surrounding surface was eroded away. From the stereomicrographs prepared, estimates were made of the difference in height between the original surface underlying the nickel and the surrounding etched surface.

After removing the specimen from the scanning microscope, the ion-etched surfaces were studied directly by incident light microscopy and by replica techniques.

Replica methods

Formvar replicas, stripped from the surface on an adhesive cellulose tape backing, were shadowed with palladium or chromium, removed from the backing tape in petroleum ether, and mounted directly on copper grids for electron microscopy. Thick (~ 3 μ) Formvar replicas were shadowed with aluminum, mounted in Canada Balsam, and examined with phase contrast light microscopy. Thick poly-vinyl-alcohol replicas were shadowed with palladium and coated with Formvar (0.2% solution in chloroform). The poly-vinyl-alcohol was then dissolved off in water and the "positive" Formvar replicas mounted directly on copper grids. Triafol sheet replicas were obtained by applying this material onto specimen surfaces which had been wet with acetone. The triafol replica was then shadowed with palladium and covered with evaporated carbon. The "triafol" was dissolved away from the "positive" carbon replica in ethyl acetate. Good replicas were obtained by this technique, although it was found difficult to mount the thin carbon film flat on the grids. Stereo-pair transmission electron micrographs were prepared from these replicas using a Siemens Elmiskop I electron microscope.

RESULTS

The nature of the surfaces produced by argon ion bombardment of enamel and dentine may be seen by reference to Figs. 3–8. Dentine was removed at a greater rate than enamel and was thus etched to a greater depth (Figs. 3 and 5). Within the dentine the tissue between the minute dentinal tubules (intertubular dentine) was etched to a greater depth than the immediately surrounding tissue (peritubular dentine (5), but it appears that the difference in height between the peritubular and intertubular dentine ceased to increase when it had reached approximately 1 μ (Figs. 3 and 8). The surface of the dentine became increasingly rough with bombardment.

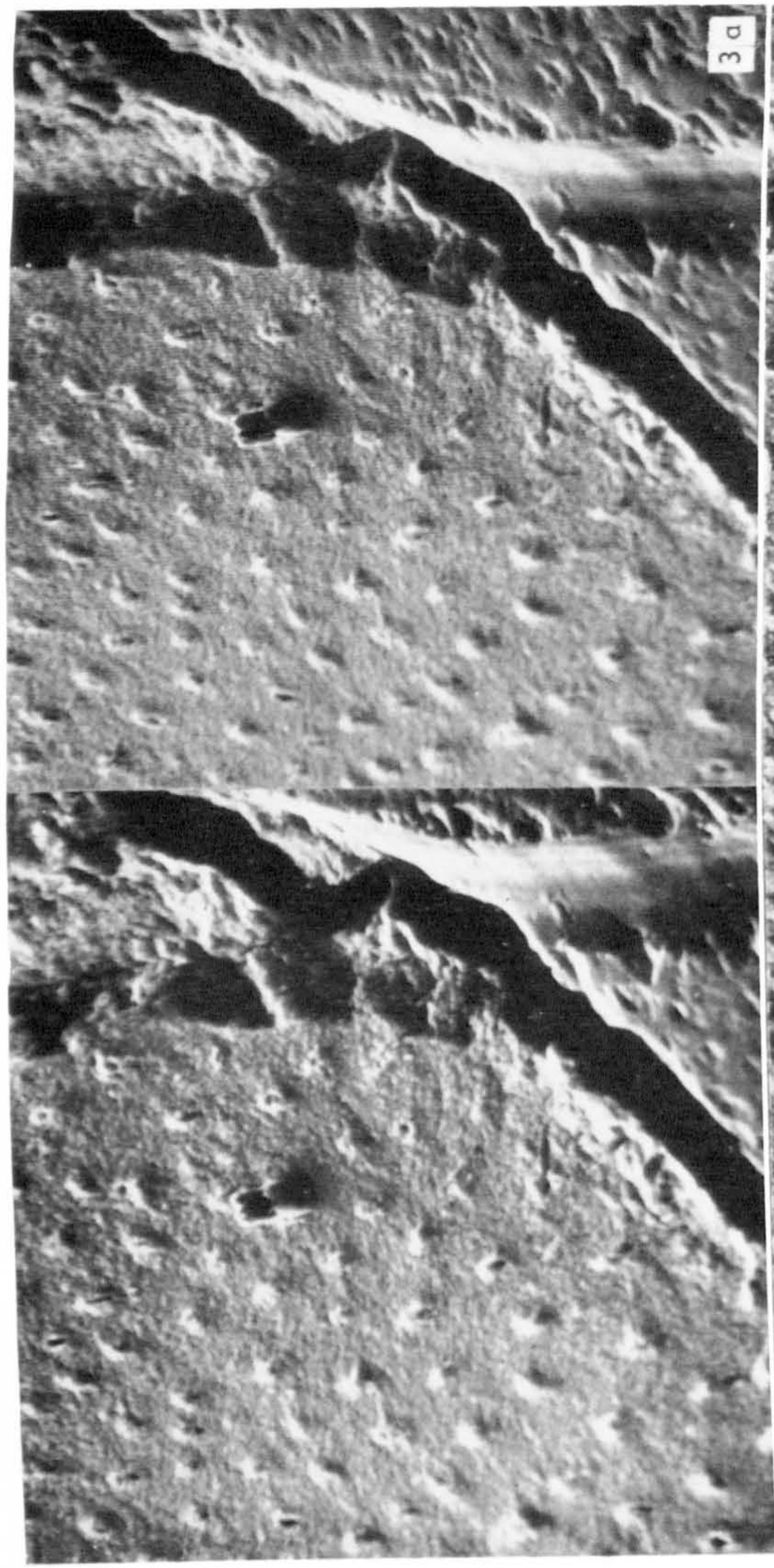
Enamel surfaces were also etched differentially. The composition of enamel is remarkably uniform throughout, but it is subdivided into units called "rods," approximately 5 μ in diameter, which are separated from one another by the so-called "inter-rod substance." The principal cause of its subdivision is a change in the crystallite orientation in the different regions. The enamel rods extend from the enamel–dentine junction to the surface of the enamel, but they are not straight; they lie in groups, such that one group or band of rods crosses over the adjacent group at an angle. The optical phenomenon produced by this aspect of enamel structure was first described by Hunter (*13*) and later by Schreger (*16*). Here, we are only concerned with these "Hunter–Schreger bands" in so far as we were enabled, by their presence, to study the effect of etching the surfaces of enamel rods which had been cut in every direction. After etching, Hunter–Schreger bands and regions called "tufts" were revealed as identifiable features at low magnifications (Fig. 5). "Tufts" are regions

List of Abbreviations

C = Crack	*J* = Enamel–dentine junction
D = Dentine	*P* = Peritubular dentine
E = Enamel	*R* = Rod
H = Hunter–Schreger band	*S* = Reference scratch
I = Intertubular dentine	*T* = Tuft

FIG. 3*a*, *b*, *c*. A sequence of stereo-pair scanning electron micrographs of the same area of human enamel and dentine. The peritubular dentine stands above the intertubular dentine. This figure shows the increasing roughness of the surface of the dentine. The tubule openings in *c* are larger than in *b* which are, in turn, larger than in *a*. The three micrographs are successively nearer to the enamel–dentine junction where one might have expected to find the dentinal tubules more completely occluded with dentine of the peritubular type. Both arms of the symbol (*b*) = 20 μ (in these directions).

(*a*) After 21.5 minutes' bombardment. The dentine surface has been etched down 8 μ below the original surface level, which is preserved under a nickel particle. (*b*) After 16.7 minutes' more bombardment the thickness of the shielding particle was reduced and the surrounding dentine etched down 12 μ below the original surface. (*c*) After 23 minutes' more bombardment the nickel particle had been etched away and the dentine "pillar" became cone-shaped.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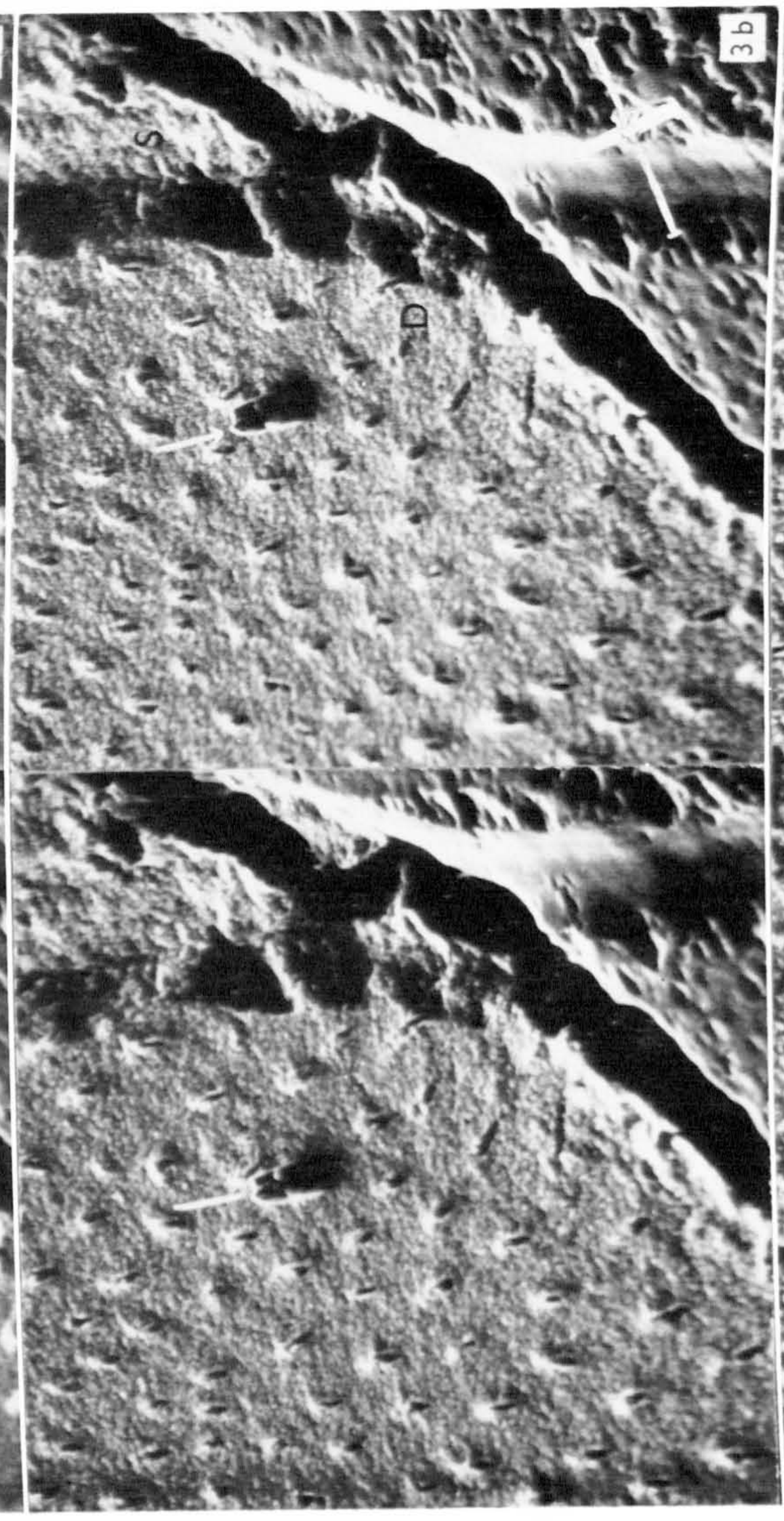
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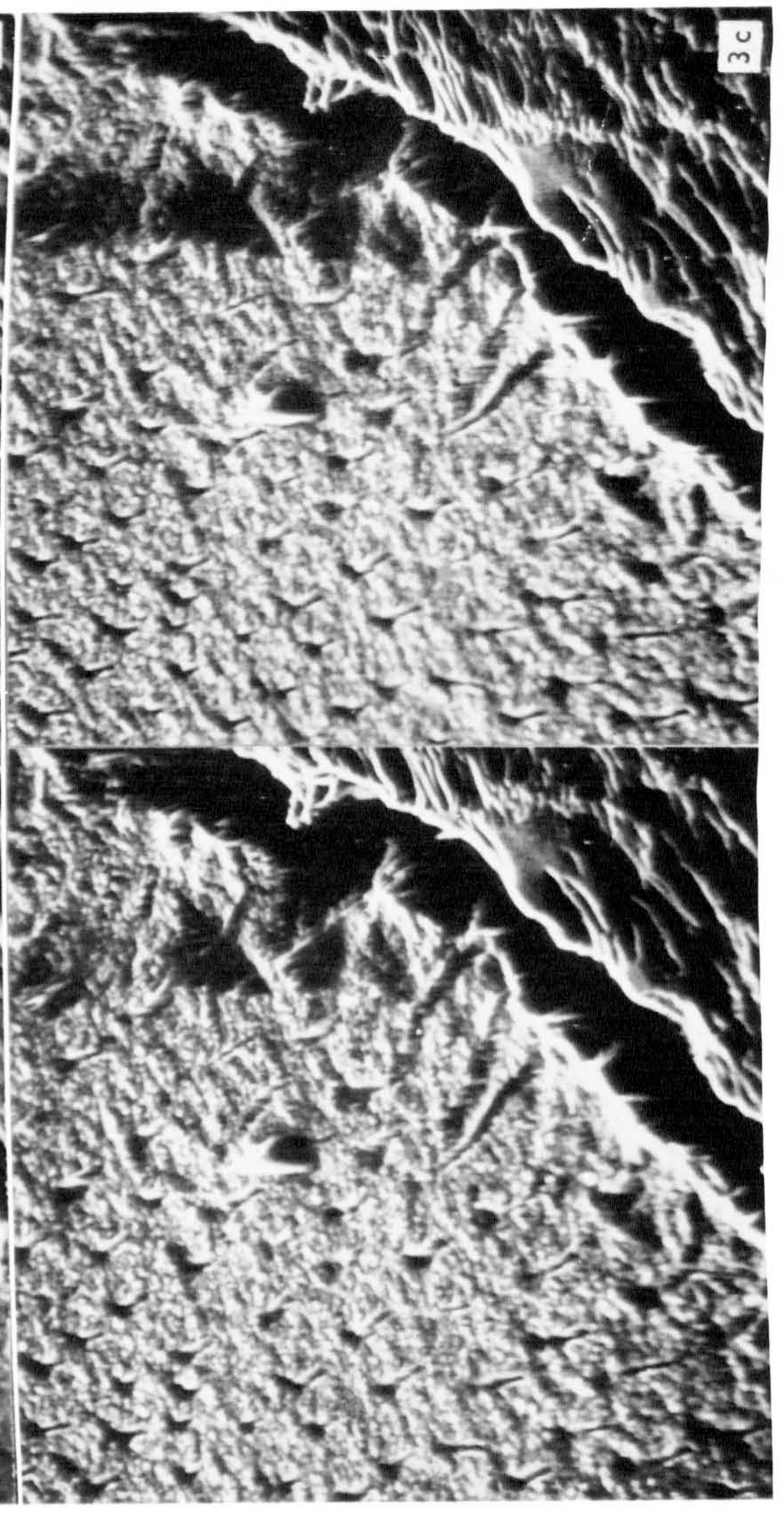
D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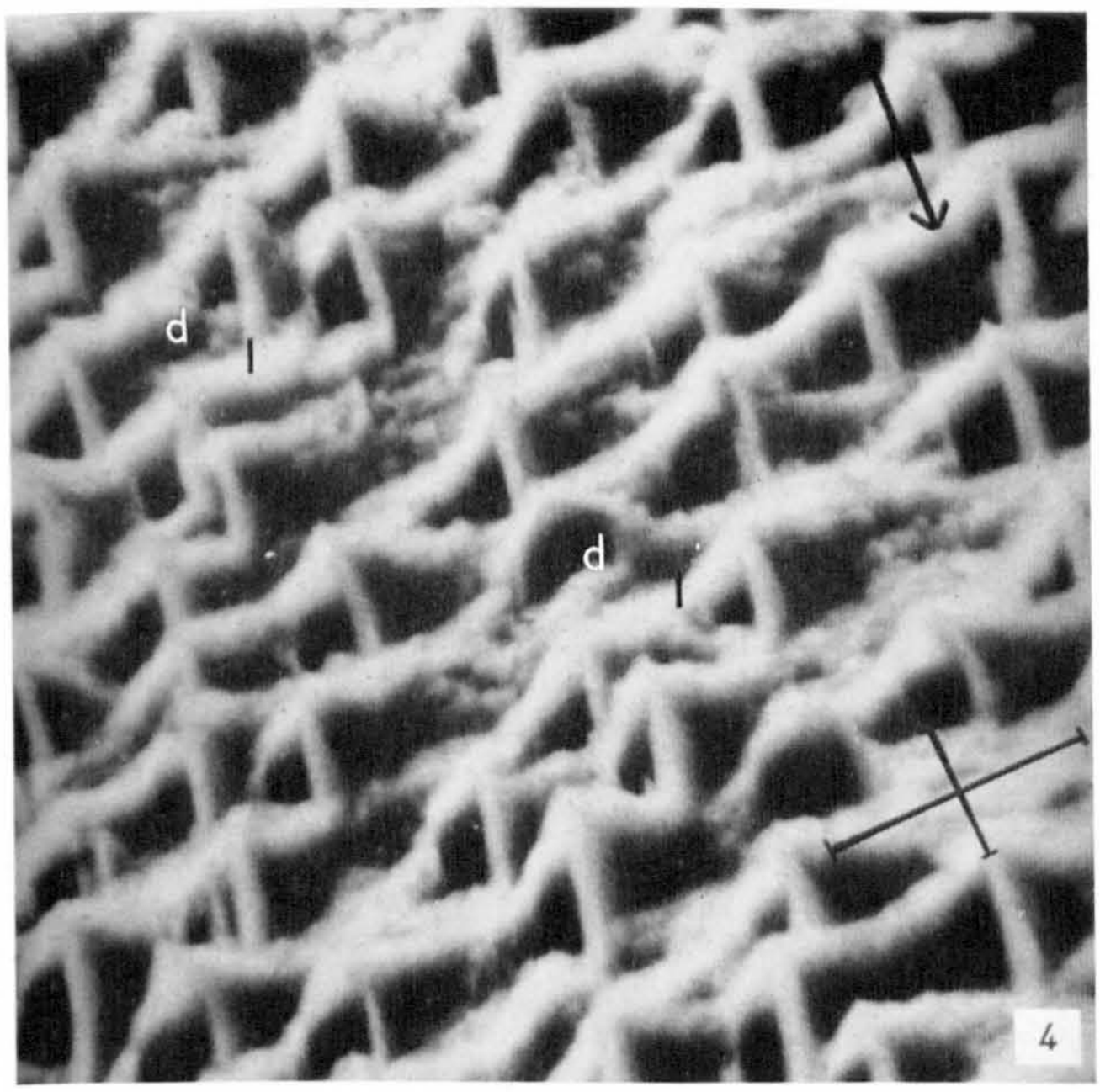

FIG. 4. Scanning electron micrograph of *Macropus* dentine (close to pulp chamber) after bombardment. The peritubular dentine is "standing proud" of the intertubular dentine. Both bars = 4 μ.

formed by groups of rods close to the enamel–dentine junction, which remain defectively mineralized after development has been completed. The "tufts" could be seen because they had been etched to a greater depth than the surrounding enamel. At higher magnifications it could be seen that the cores of those rods which had been sectioned transversely were eroded to a greater depth than the inter-rod substance.

DISCUSSION

It is clear that we caused an alteration in dried enamel and dentine surfaces by bombardment with argon ions. The rate at which material is removed from a surface under ion bombardment may be correlated with the underlying structure, since this rate might be expected to depend on differences in mineralization, and, in a region of uniform mineralization, on differences in crystallite orientation.

Dentine and enamel have the same inorganic crystallite component—calcium phosphate, with crystallographic properties widely held to be those of hydroxyapatite —whereas their protein matrices have important chemical differences. Nevertheless, because of the similarities in their atomic composition we would still expect the etching rate of the different protein matrices to be much closer to each other than to

FIG. 5. Scanning electron micrograph of human enamel and dentine after bombardment. Both bars = 40 μ.

that of hydroxyapatite. Therefore, to simplify the discussion, we take dentine and enamel to consist of hydroxyapatite with admixtures of organic matrix in different proportions.

The differences in erosion rates which we observed, that might be ascribed principally to such differences in the degree of mineralization are:

(1) tuft enamel is eroded more rapidly than the surrounding normal enamel. "Tufts" are known to be regions of defective mineralization.

(2) Dentine is etched away more rapidly than enamel. Enamel is known to be more highly mineralized than dentine.

(3) The intertubular dentine is etched away more rapidly than the peritubular dentine. Peritubular dentine has been shown to be more highly mineralized than intertubular dentine (*2*) but, in this case, it is also possible that differences in the orientation of the crystallites may have contributed to the differences in etching rates.

Since it is known that sputtering rates vary with crystal orientation, differences in level of the ion-etched surface in an area of uniform composition may be related solely to differences in crystallite orientation. Enamel was the only region examined in these experiments where this mechanism is likely to have been predominant.

In the etched surfaces of enamel sections we observed differences in level which are probably related to differences in hydroxyapatite crystallite orientation. If this is so, it may be that there are some points about the orientation which have not been adequately stressed by previous workers. For example, it appears that the rod substance interdigitates with the inter-rod substance to a large extent. Along the length of the rods we noticed variations in the surface level which occurred at regular intervals—these intervals being approximately equal to the width of the rods. We presume that these variations correspond to the cross-striations of the enamel rods seen with the optical microscope. Our present experiments indicate that these cross-striations are due to either a periodic variation in the width of the rods, or to a periodic minor change in the crystallite orientation along the length of the rods, or to both these factors. This view finds support from the observations of Helmcke *et al.* (*11*, *12*) who reached a similar conclusion on the basis of a replica study. On the surface of some of the transversely sectioned rods, etching produced a characteristic deformation that took the form of a concave slope from one side of the rod to the other, with an abrupt rise near the adjacent rod. This deformation, which was best seen in the replicas, might indicate a gradual change of crystallite orientation from one side of the rod to the other. This evidence would support the hypothetical pattern of crystallite orientation suggested by Poole and Brooks (*14*). In all those regions where we produced prominent relief on the surface, it is possible that the final profile was in part determined by the redeposition of sputtered material, although we have no experimental information about the directions of emission of the sputtered material, or of the manner in which it is redeposited.

Ion etching, using the apparatus described, has some general advantages over

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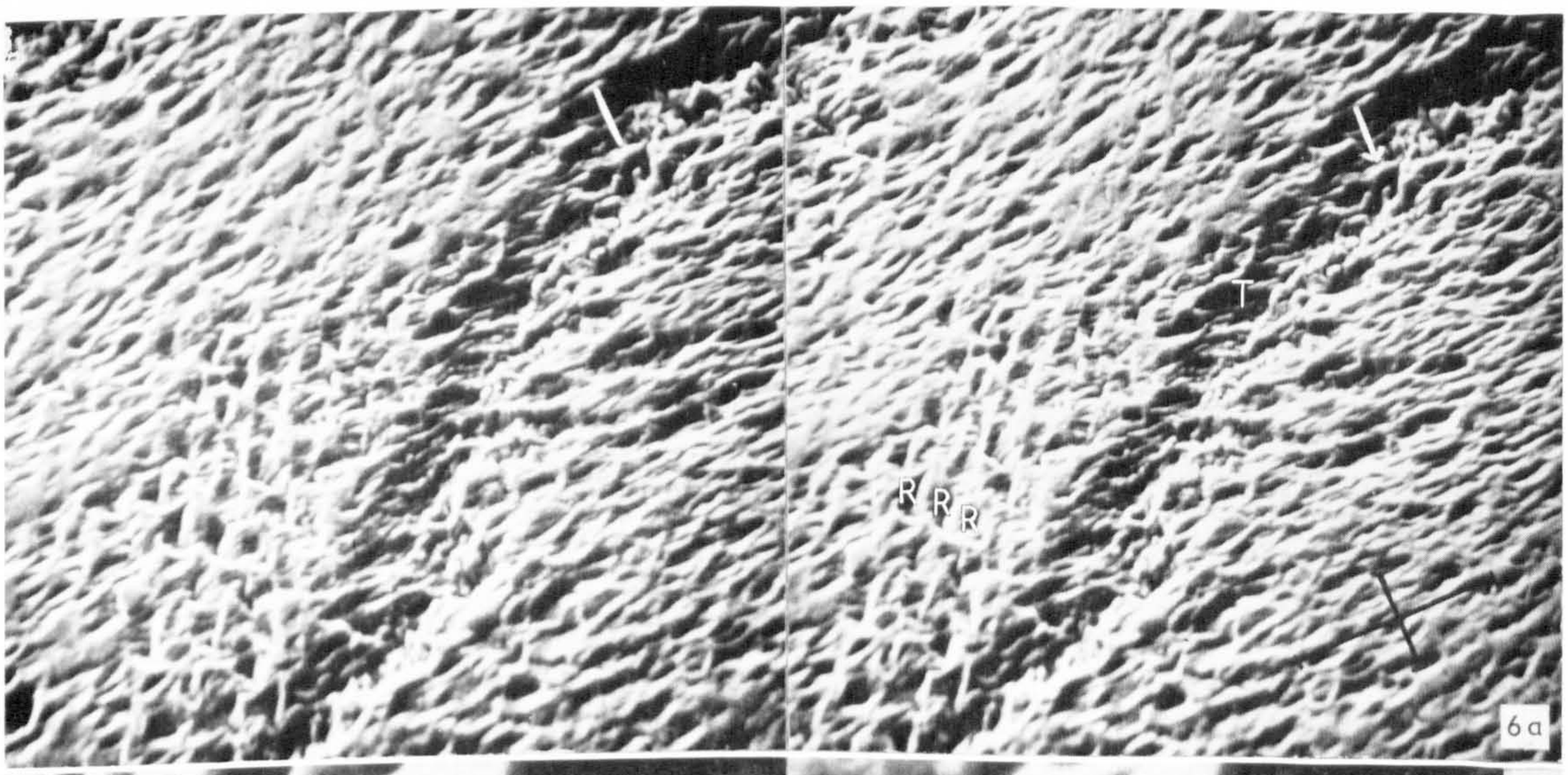
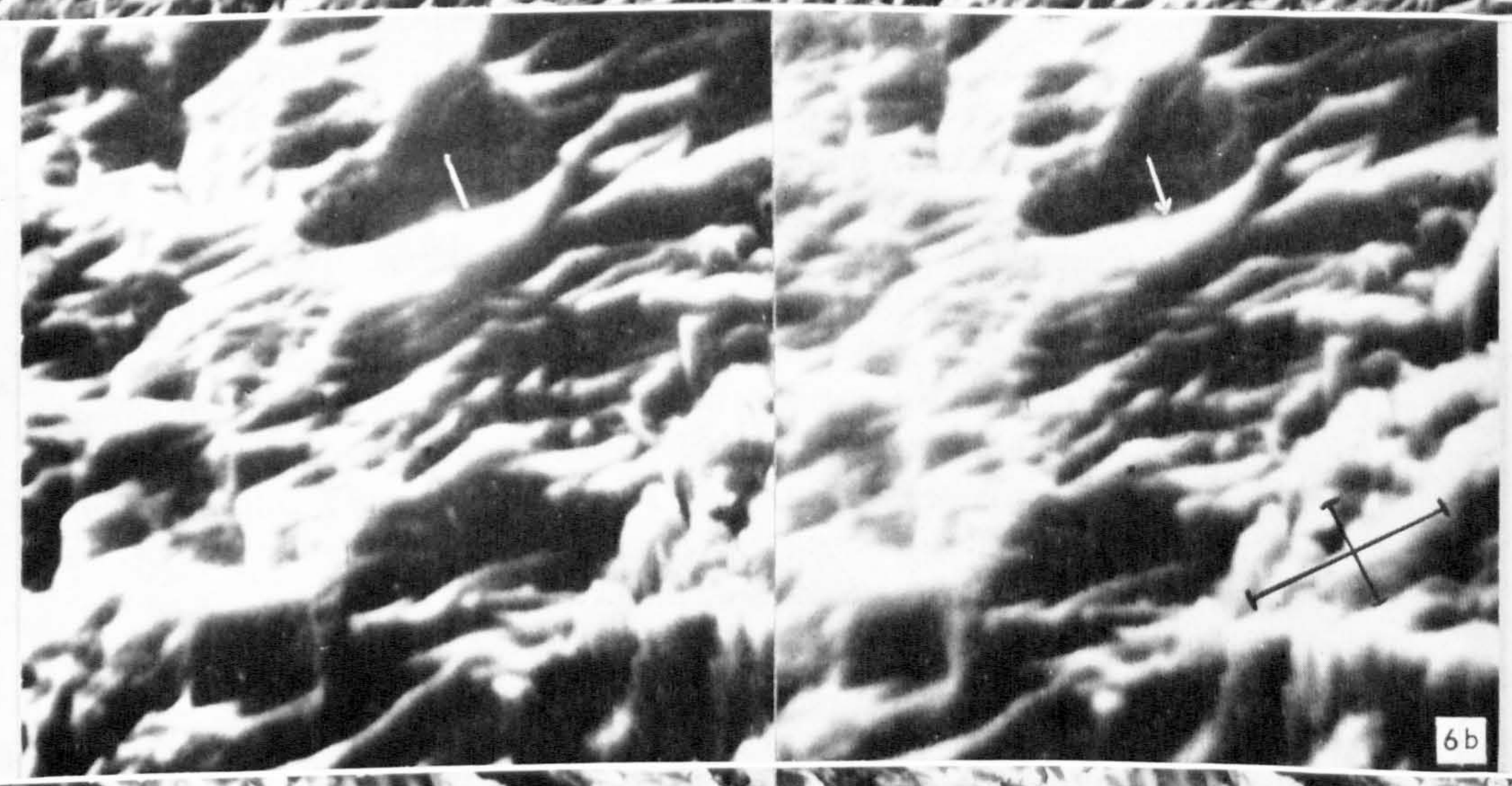
FIG. 6*a*, *b*. Scanning electron micrograph of human enamel after bombardment. (*a*) A "tuft" region can be seen etched deeper than the surrounding enamel. Both bars = 20 μ. (*b*) Center of same field. Both bars = 4 μ.

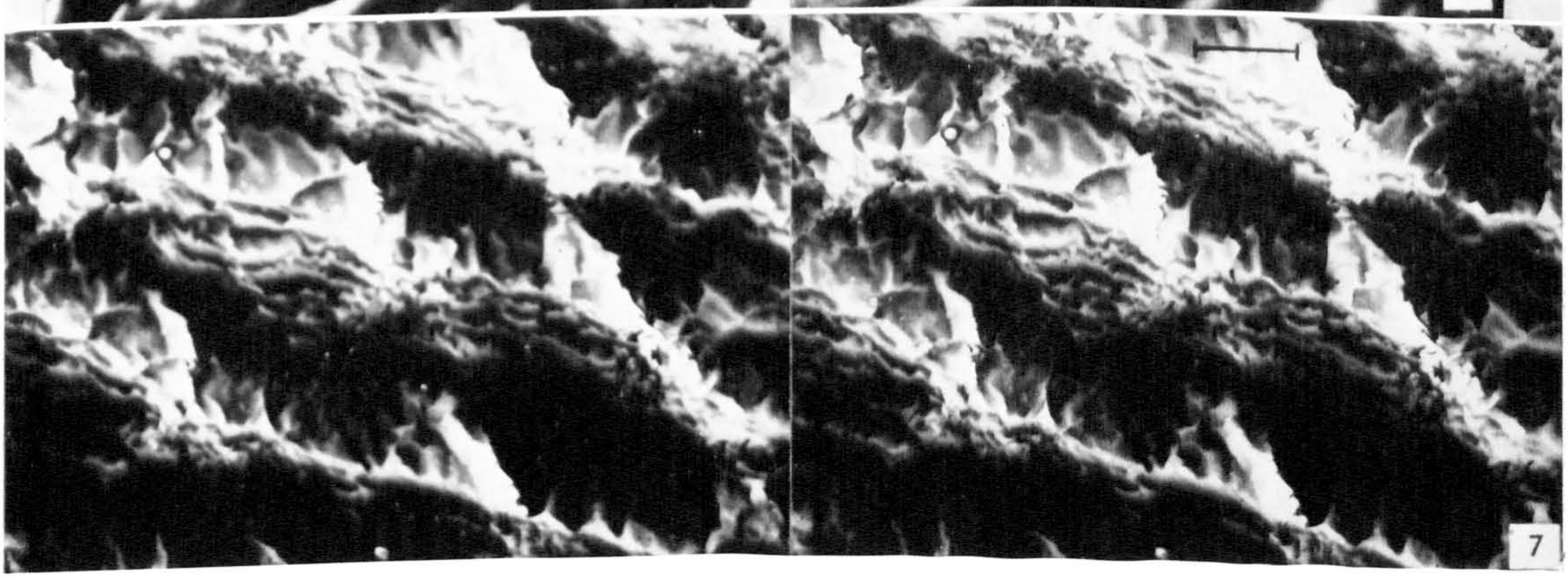
FIG. 7. Transmission electron micrograph stereo-pair prepared from palladium-shadowed Formvar replica of *Macropus* enamel; after etching. Bar = 4 μ.



6a



6b



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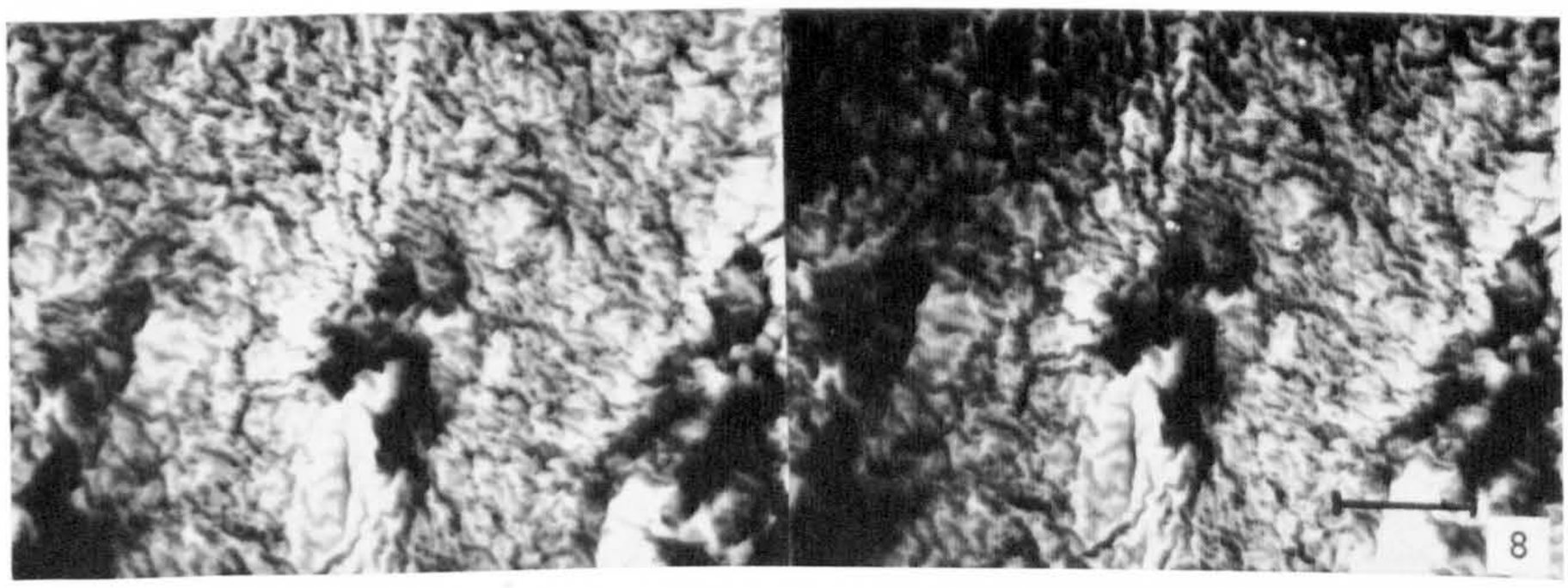

FIG. 8. Transmission electron micrograph stereo-pair prepared from palladium-shadowed Formvar replica of human dentine; after etching. Bar = 4 μ.

etching by chemical solutions. Some of these advantages lie in the nature of this etching process and others in the design of the scanning electron microscope. Apart from the composition and structure of the surface under bombardment, the variables which determine the rate of etching are the atomic number of the ions and the voltage and angle of incidence of the ion beam: these can be accurately measured and controlled.

The depth of etching can be calculated from measurements made on stereomicrographs of areas in which the original surface level has been preserved under an adherent shielding particle, as described above. These micrographs are prepared, of course, without removing the specimen from the scanning electron microscope. For future work it would be preferable to use shielding particles of vanadium, tantalum, or some other material with a very low sputtering coefficient, instead of nickel. This would allow two to three times as much material to be removed before the shielding particles were eroded away themselves.

The micrometer specimen stage controls of the scanning electron microscope enable one to relocate quickly, to within 1 μ, any previously observed area, and hence we were able to study the erosion of many areas at high magnification and rebombard them until the optimum degree of etching had been achieved. This allowed changes at successive times and depths to be studied.

The scanning electron microscope has a much greater depth of focus and a better resolution than the optical microscope. It has an additional advantage in the study of light transparent surfaces, since the scanning electron micrsocope image is determined by only a thin layer ($\sim$ 100 Å) of the specimen surface.

In ion etching, only the surface is attacked, whereas acid solutions, for example, penetrate to some depth and loosen crystallites in the surface so that these come away with the replicating material, producing a "pseudoreplica." We have found that ion

etching produces a clean, hard surface which replicates well and there is no visible damage even after many replicas have been taken.

In spite of the limited resolution (400 Å) of the scanning electron microscope, the instrument proved of great value, because it enabled us to follow the sequence of changes during bombardment and this greatly reduced the time taken to develop the technique.

Ion etching, with parallel ion beams, appears to be of value as a preparatory procedure in the study of mineralized tissues. It accentuates differences in structural composition at a surface. In areas of uniform composition it reveals differences in crystallite orientation.

To further the interpretation of the results that can be obtained by the ion etching of the mineralized tissues of vertebrates it will be essential to have experimental data on the variation of the sputtering coefficient of an apatite crystal with the angle of incidence of a given ion beam to the surface and the crystal lattice. It would be convenient to use a 5 kV argon beam, as this has proved to be a good compromise between a number of conflicting requirements.

A.B.: Financial assistance was received from the Advisory Medical Research Committee and Yarrow Research Funds of the London Hospital and Medical College, some ancillary apparatus is on loan from the Medical Research Council, and the Siemens Elmiskop I is on loan from the Wellcome Trust.

A.D.G.S.: The scanning electron microscope with attached ion beam equipment was developed in the Engineering Laboratory of the University of Cambridge under the surveillance of Professor C. W. Oatley, for research on ion etching. This work was made possible by U.K.A.E.A. Contract No. 13/5/165/1066.

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Application of the Scanning Electron-probe X-ray Microanalyser to Dental Tissues

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The scanning electron-probe X-ray microanalyser utilizes the property of X-ray emission which results from the bombardment of elements by an electron beam. The instrument provides qualitative and quantitative data which can be related to the topography of the surface of the spectrum. Thus, it is possible to identify an element and to obtain an accurate estimation of the quantity of this element in the surface of the sample. The present instrument is capable of identifying elements having atomic numbers greater than ($Z = 12$).

Polished cut surfaces and sections of human and rodent teeth were examined using this instrument. In rodent incisors a clear identification of iron in the enamel can be obtained. In human dentine differences in the quantity of calcium present in different regions can be demonstrated. Quantitative assessment of elements in biological material provides, however, special problems of interpretation. The experimental evaluation of these problems is discussed.

Important advances in knowledge of biological systems might be expected if the normal and experimental incorporation of elements into biological structures could be studied *in situ* at a microscopic level. The scanning electron-probe X-ray microanalyser described by Duncumb and Cosslett (*1*) (Fig. 1) was originally designed for micro-quantitative analysis in metallurgy. Some preliminary experiments in the preparation and examination of mammalian teeth have now been made in order to find out whether this instrument can also be usefully applied to the study of mineralized biological systems.

The scanning electron-probe X-ray microanalyser

The instrument utilizes the property of X-ray emission which results from the bombardment of elements by an electron beam. The electron beam from a heated tungsten filament is accelerated by a potential which can be continuously varied from 1–50 kV. This beam is focused onto the surface of the specimen as a spot less than 1 μ in diameter. By means of deflecting coils the spot may be scanned over the

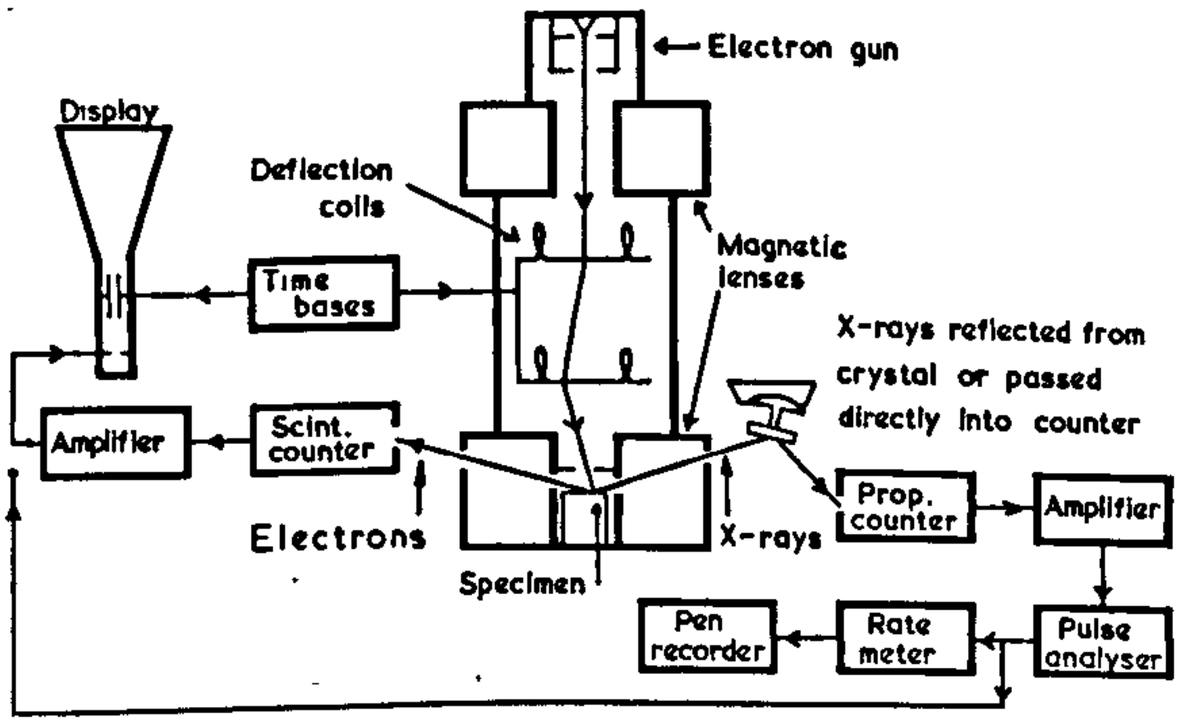

FIG. 1. Diagram of the scanning electron-probe X-ray microanalyser.

surface in a square raster of variable side up to 0.5 mm. Electrons scattered from the surface excite a phosphor and the light produces an electrical signal from a photomultiplier tube which is used to modulate the brightness of a cathode ray tube. The spot of this tube is scanned in synchronism with the spot on the specimen, so that an image of the surface is built up on the long-persistence cathode ray screen. The magnification of the image, which may be varied, is the ratio of the side of the screen to the side of the area scanned. Contrast in the image is determined partly by the physical form of the surface and partly by its elementary composition. This electron image is primarily used for identifying regions of interest already selected under the optical microscope.

The electrons striking the specimen excite a spectrum of X-rays in which are lines characteristic of the elements present. These are reflected by the curved crystal of the spectrometer which enables a given line to be selected. The intensity of the emission can be measured by a gas flow proportional counter. The signal from this can be used to form an image on the screen, showing the distribution of the element selected. Elements from magnesium ($Z=12$) to uranium ($Z=92$) may be detected in this manner.

The scanning may be stopped and the electron-probe placed on any desired feature of the specimen by manual positioning of the display tube spot on the electron image afterglow. Quantitative analysis may then be carried out with the help of a ratemeter or scaler in conjunction with the X-ray spectrometer. The intensity of the characteristic emission of an element is, to a first order, proportional to its concentration. The analysis may be carried out on a single spot by rotating the spectrometer crystal and recording the X-ray intensity as various wavelengths are reflected. The elements present produce peaks above the background of noise and continuous X-radiation. Alternatively, the crystal may be set to reflect the radiation from an element of interest and the distribution of this may be recorded as the spot is moved slowly across a

given line on the specimen. If the electron-probe is made to scan this line rapidly, the line can then be moved bodily across the specimen, thus recording average variations in the concentration of the elements. This method was used in the present experiments to demonstrate the difference in calcium concentration in the enamel and dentine layers, by orienting the line parallel to the enamel–dentine junction, and observing changes of intensity of the Ca Kα radiation as different regions of the tooth were scanned.

MATERIAL AND METHODS

The specimen holder was designed to take cylindrical rods or discs $\frac{1}{8}$ inch in diameter. The end of the rod intended for examination must be well polished, since the depth of focus is small and as the X-ray emission is collected at a shallow angle, surface projections may cause "shadowing." Polished surfaces satisfactory for examination were obtained from alcohol-fixed or fresh, unfixed rodent incisors and human teeth, some after embedding in methylmethacrylate and others without embedding. In some cases ground sections were prepared by first cutting through selected regions and then polishing in the normal manner. The region of the section selected for study was then cut out using a steel trephine of the appropriate size. In other cases rods were turned down on a lathe. The end of the rod was then ground and polished to a highly finished surface using a perspex jig to hold the small rod against the surface of the lap of a metallurgical polishing machine.

It was soon found to be necessary to coat the polished surface of the specimens with a thin layer of aluminum by vacuum evaporation, in order to provide a surface which is able to conduct away electrostatic charges, which otherwise build up on nonconducting materials subjected to electron bombardment.

Ultrathin sections suitable for electron microscopy (approximately 1000 Å thick) were cut with a diamond knife and mounted on electron microscope grids. These were also coated with aluminum before examination but, although the instrument could image these sections, quantitative results could not be obtained since the electron beam was not stopped completely by such a thin specimen.

In order to be reasonably sure of obtaining a fair differentiation of tissue elements in our preliminary experiments, the pigmented surface of the rodent incisor, which is known to contain iron (*4*), was selected as a suitable test region to be studied. Methacrylate-embedded rat incisors (*Rattus norvegicus*) were turned down into rods in the way described, so that after grinding and polishing the longitudinal cut surface of the pigmented incisor enamel was situated across the maximum diameter of the circular rod end. Human dentine was selected for the second type of test specimen, because regional differences in mineralization which may be related to age changes or due to a reaction of this tissue to wear, are known to occur in the walls of the minute dentinal tubules which traverse the whole depth of the dentine. In this case rods of dentine were prepared and the tissue oriented in such a way that the final surface was normal to the direction of the dentinal tubules.

RESULTS

The topography of the ground surface of the rodent incisor is clearly demonstrated by the image formed on the cathode ray display tube by the scattered electrons (Fig.

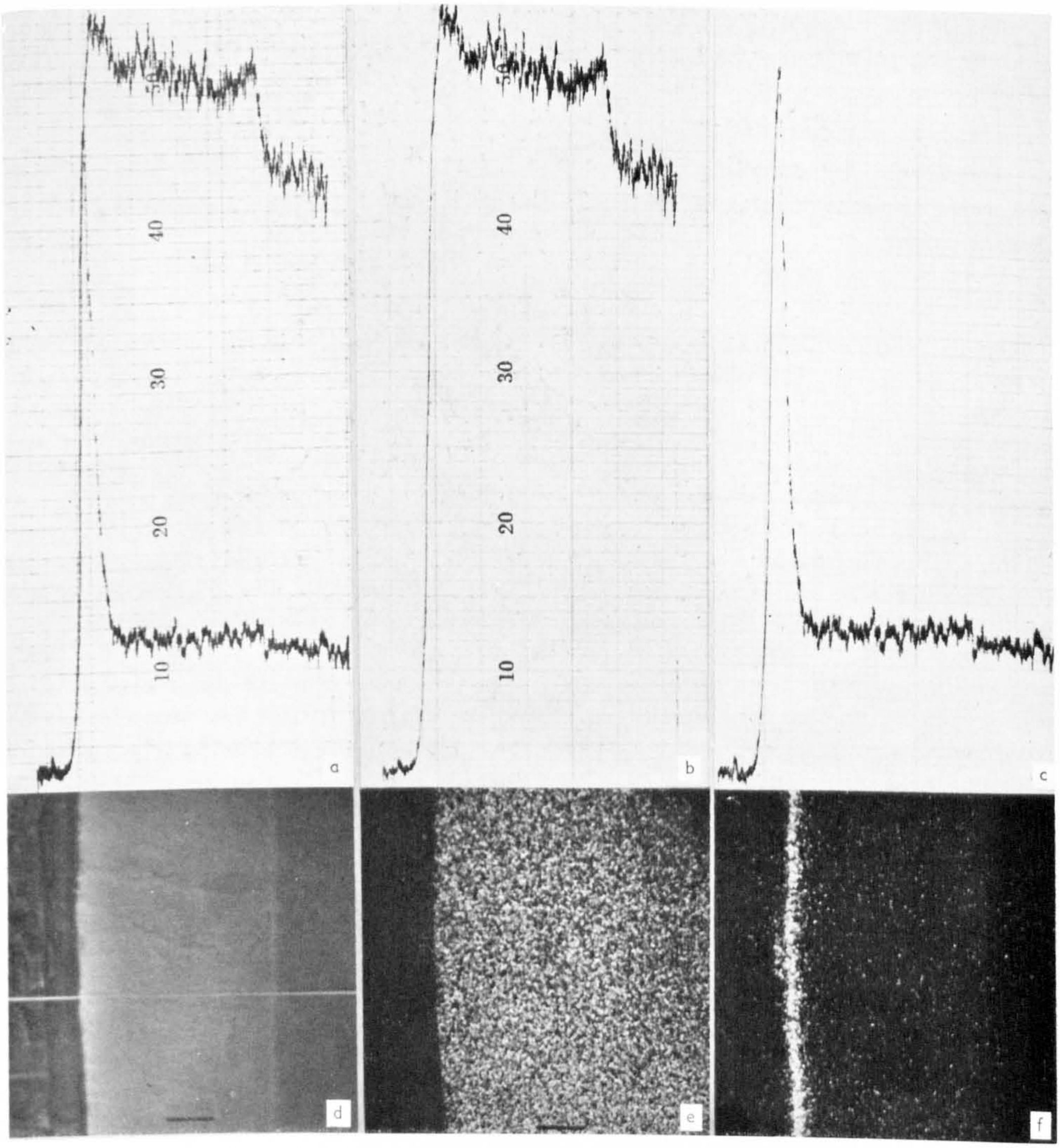

FIG. 2. (*a*) Superimposed Ca Kα (*b*) and Fe Kα (*c*) emission traces, both taken by moving probe, rapidly scanning a line parallel to enamel–dentine junction in rodent incisor while recording selected X-ray emission intensity. (*b*) Ca Kα line trace. (*c*) Fe Kα line trace. (*d*) Image formed by reflected electrons. (*e*) Image formed in Ca Kα radiation. (*f*) Image formed in Fe Kα radiation. Bar = 50 μ.

2*d*) and images formed by the Ca Kα (Fig. 2*e*) and Fe Kα (Fig. 2*f*) emission can be readily related to the landmarks on the specimen. Measurements of the Fe Kα emission from the surface zone of the tooth and a standard sample of iron were made by means of a ratemeter. In the specimen studied the concentration of iron at the surface of

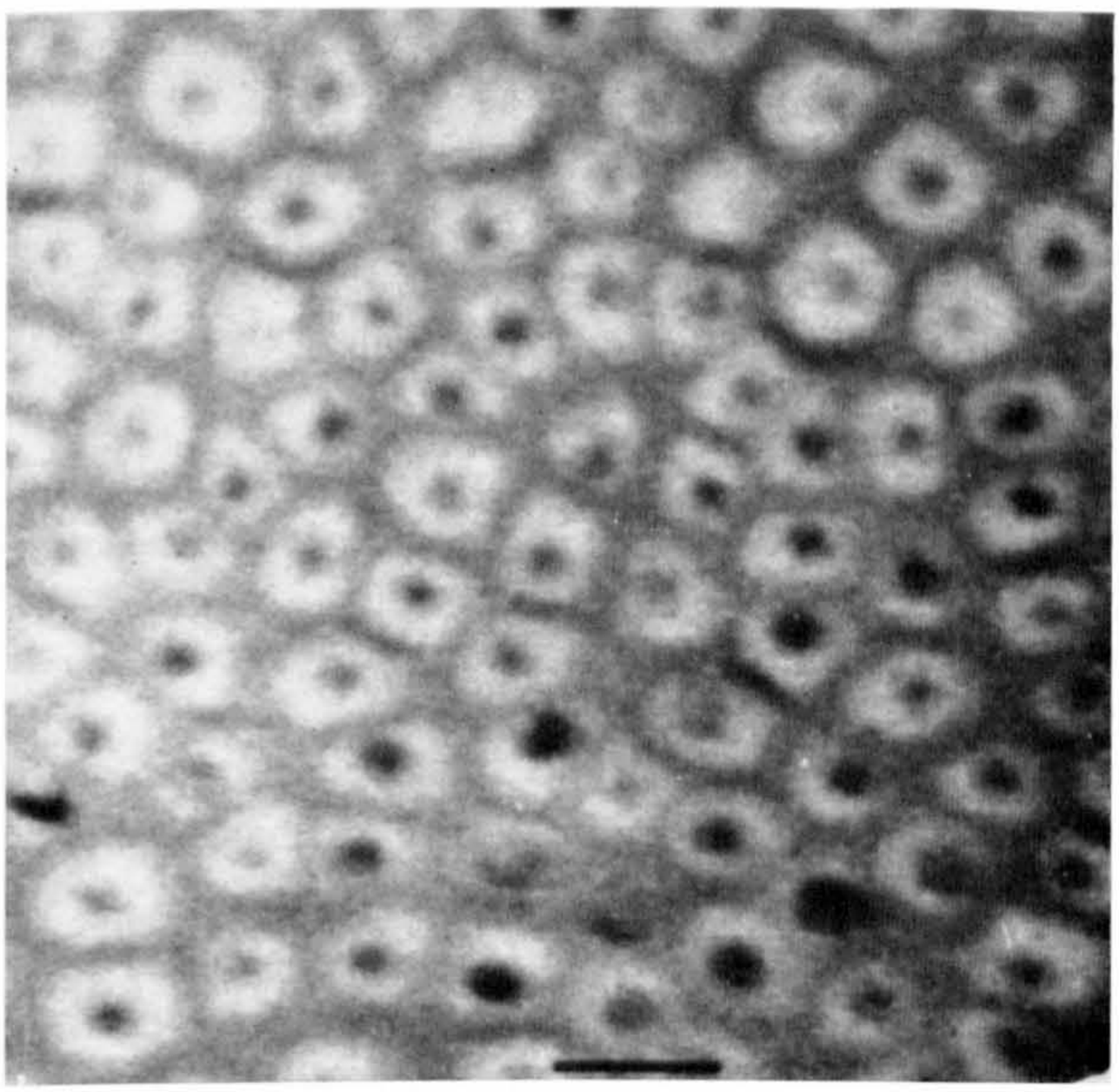

FIG. 3. Electron image of human dentine. Plane of surface transverse to the dentinal tubules. Bar = 5 μ.

the tooth was found to be 7.6% of that of the pure sample. By superimposing the Ca Kα and Fe Kα pen traces (Fig. 2*a*, *b*, *c*) obtained from the rodent incisor, it was found that the Fe Kα emission reaches a peak at a position where the Ca Kα emission is beginning to fall. It would seem, therefore, that the iron is not a discrete layer on the surface of the enamel but that calcium and iron are intimately mixed. This observation confirms those obtained by histochemical techniques (*4*), and has the advantage that the method is free from diffusion artifacts which are a notorious limitation of histochemistry.

The results obtained from the human dentine specimens were equally encouraging. The electron image of human dentine in Fig. 3 shows up quite distinctly structural differences in the peritubular region (*2*). In this specimen the intensity of Ca Kα emission in the peritubular region was found to be 70% of that of a pure sample of hydroxy-apatite, and 60% in the intertubular dentine.

DISCUSSION

The visual resolution of the X-ray image of the specimen on the fluorescent screen is poor compared with the optical and the electron resolution. This appears to depend on the coarse grain of the fluorescent screen, background noise and of course,

the magnitude of the variation in the quantity of an element in the polished surface of the specimen. Pen-recorder traces of X-ray emission intensity, which can be accurately related to the electron image of the specimen surface, reveal the true resolving power of the instrument and yield much more precise data. However, it should be mentioned that the resolution here is limited slightly by the depth of electron penetration and scattering within the surface layers of the specimen. The magnitude of this effect will vary with the nature of the specimen under examination and in the case of bones and teeth probably varies with the degree of mineralization. Thus, a small variation in the quantity of, for example, calcium in a very small area (approximately 1 μ diameter) may not be readily related to the finer surface details of the specimen.

In order to obtain quantitative information, the counts obtained from the characteristic emission of the element are compared with a standard pure sample of the element being studied. This data provides useful and meaningful information for metallurgical specimens when expressed as a percentage of the sample. However, some care must be exercised in interpreting the meaning of results obtained in this way on biological specimens. For example, quantitative variation in the Ca Kα emission of the dentine sample presents some difficulties of interpretation. Bone, dentine, and enamel are composed of extremely small crystallites of hydroxy-apatite embedded in an organic matrix, and for this reason the choice of hydroxy-apatite as the standard reference was made. The depth of penetration of the specimen by electrons varies with the density of the material. In the case of a specimen consisting of materials of different densities, X-ray emission will result from atoms situated at different depths. In the case of the hydroxy-apatite standard a quantitative value is obtained from a close-packed crystalline material which has a density of 3.21 g/cc, the density of enamel, however, is between 2.7 and 3.0 g/cc and dentine 2.0–2.4 g/cc (*3*). These density differences are, of course, due to the admixture of the organic matrix and water to the hydroxy-apatite. In a comparison between enamel and dentine, X-ray emission will be excited from a greater volume of dentine than of enamel, since the emitting calcium atoms of dentine are more widely separated by this organic material. If X-ray emission results from different volumes of the different parts of a specimen being analyzed, then the results are not comparable unless this difference in volume is taken into consideration. It is possible that this difficulty can be overcome by removing the organic material and water and replacing them with a material having the same density as hydroxy-apatite but not containing calcium. In this case one might expect the depth of electron penetration and electron scatter to be uniform, thus exciting X-ray emission from a surface layer of uniform depth. Differences in Ca Kα emission would then be due only to differences in concentration.

At present, however, a comparison between the mineralized tissue and the hydroxy-

apatite might reveal quantity differences which would be related to the degree of mineralization (packing of crystallites). As far as the experiments have taken us, this does seem to be the case, since the differences in the mineralization between the peritubular and intertubular dentine are, in all probability, due to differences in the packing of hydroxy-apatite crystallites in an organic matrix.

It is quite clear from the preliminary experiments that much useful data can be obtained from mineralized tissue by the application of the scanning electron-probe X-ray microanalyser. It opens up the possibility of obtaining quantitative and qualitative element analysis of differences in the surface zone of tooth enamel, and introduces the possibility of studying changes in this zone that may occur with advancing age. Furthermore, early stages in fossilization and early changes in enamel caries might also be profitably studied.

We are indebted to Dr. G. F. Klaringbull and Mr. P. G. Embrey, Department of Mineralogy, The British Museum (Natural History) for supplying us with samples of hydroxy-apatite. Our thanks are also due to Dr. V. E. Cosslett, the Cavendish Laboratory, Cambridge, and Dr. P. Duncumb, Tube Investments Research Laboratories, for their help and permission to use their illustration (Fig. 1). We also wish to thank Mr. P. Richards and Mr. R. Adams for technical assistance.

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Device for the Trimming of Blocks for Ultra-microtomy

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A single device for the precise trimming of blocks to the desired plane of section is described.

For ultra-microtomy it is necessary to have a cross-sectional block area of very small dimensions. Blocks of resin-embedded tissue have commonly been trimmed with a razor blade leaving the region to be sectioned at the apex of a pyramid. This is often difficult to achieve accurately free-hand. A simple device for trimming resin blocks accurately to the plane of section required, before attaching the block to the ultra-microtome, is particularly useful when it is desired to pre-determine the region from which sections are to be cut with a high degree of accuracy. Maas (*1*) described a mechanical device with which he was able to trim blocks to within a few micra of the selected region, using a stereo-binocular microscope and a special arrangement of optics to observe the knife cut.

The instrument described in this paper is capable of trimming blocks for ultra-microtomy to the same limits of precision described by Maas, but has the advantage that it is cheap and simple to make. It consists of two parts which can be used on any standard optical microscope.

The resin block (*G*) is mounted in a specimen holder (*H*) with a square base (see Fig. 1) similar to that designed for use with the Sjöstrand microtome.

A piece of $1\frac{1}{2}''$ brass rod was turned out to fit over the nose of a 2/3″ objective (*A*), and fitted with a screw (*B*), to hold it on the objective. The lower rim of the brass was slotted to take an injectomatic razor blade (*E*). This is held in place with two screws (*D*). The slots are arranged such that the blade crosses nearly in the centre of the field of view just above the focal plane of the objective lens. The blade slots are set at an angle of 14°, to give a cutting clearance angle of 7°.

The specimen holder containing the resin block to be trimmed is held in a heavy brass block (*I*) by a screw (*F*) so that it is 45° to the horizontal. The brass block can be held in the mechanical stage (*J*) of a microscope. The illustration shows two addi-

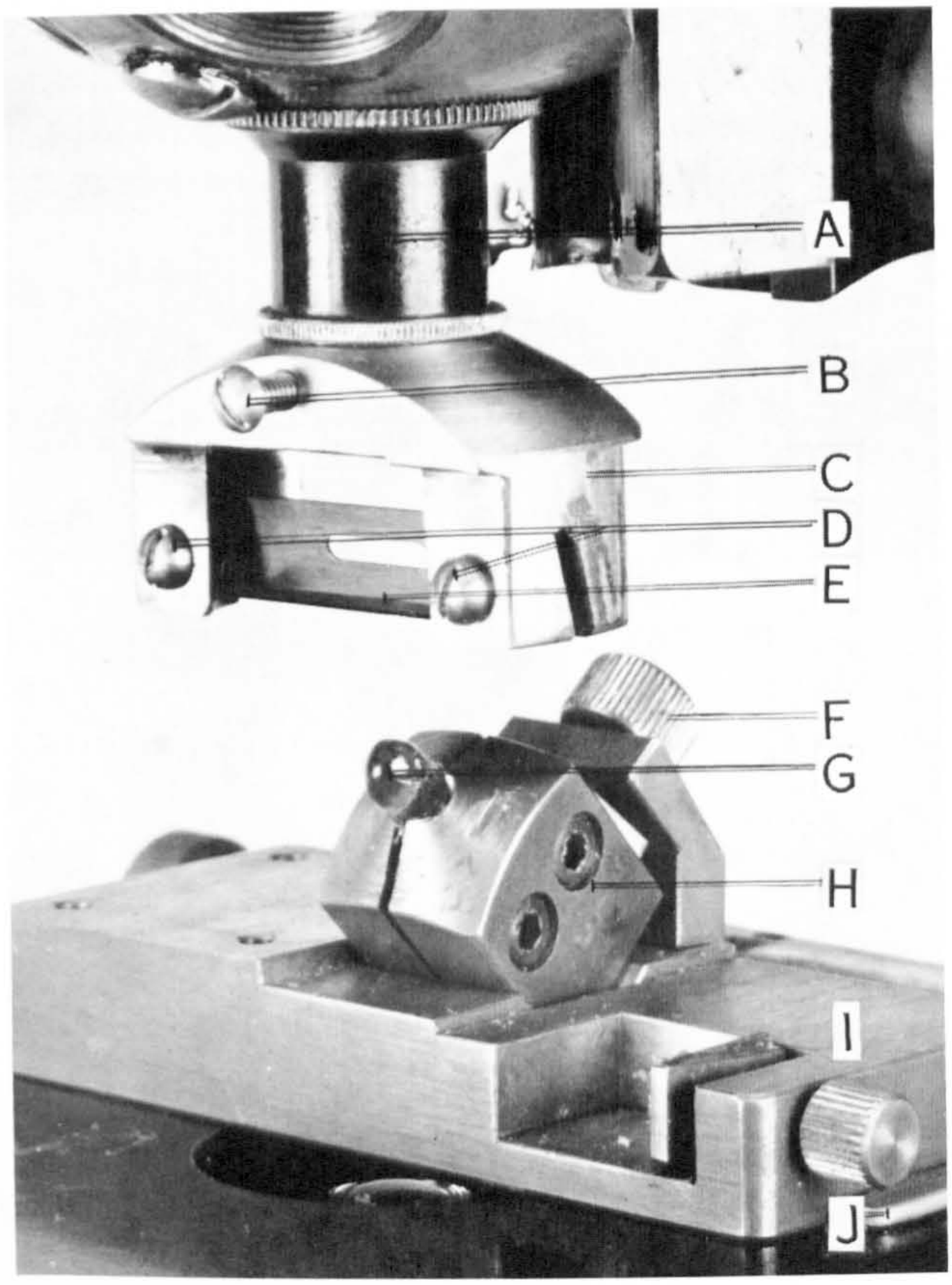

FIG. 1.

tional positions on the brass block (*I*) where the specimen holder may be stood upright or clamped horizontally, respectively.

The specimen is placed under the blade, and the tube racked down, thus cutting the specimen. The process is repeated for the four sides of the specimen, until the area to be sectioned is left at the apex of a neat pyramid. The amount to be trimmed off the block can be seen directly within the field of view and the cut aligned accurately, since the specimen comes into focus just before the blade edge is brought into contact with it. By continually advancing the specimen under the blade, the apex of the pyramid can be positioned to contain the portion of tissue that it is desired to section. Both the mechanical stage vernier, and an eyepiece micrometer can be used to measure the amount to be trimmed off the block on any one movement. The slackness normally

present in the rack and pinion of the microscope when it is racked down against opposition can be eliminated by turning to one end of the fine adjustment mechanism. Failure to make this adjustment will cause the blade to jerk through the specimen.

Methacrylate- and Araldite-embedded tissue blocks have been trimmed routinely with this device.

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Problems Associated with the Preparation of Biological Specimens for Microanalysis

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Many biological specimens can be prepared by very simple techniques for microanalysis with the electron-probe, particularly when only qualitative information is required about the distribution of elements. The same is true if the data can be accepted in the form of the mass of the element being analyzed per unit mass of the sample, that is, if the interest centers on the ratio of one element to another. In this case the principal limit to the accuracy of the data may be imposed by the available instrumentation, in that changes may occur in the specimen (e.g., thermal damage or a shift of the probe position) between recording a statistically significant number of counts for two elements in the same position in the specimen. However, there are many occasions on which it is desired to have the data in the form of the mass of the element being analyzed per unit volume of the sample. On these occasions it is the specimen preparation problem which principally limits the accuracy of the method.

A problem arises because one can neither assume a density for the specimen nor can one assume that the specimen is uniformly dense. Variations in elemental composition with biological tissues are associated with significant differences in density and, therefore, with the volume analyzed. The variations in density might be as large as 3 to 1 in the case of mineralized tissue and are due partly to differences in the atomic composition of the "bone salt," but to a great extent they are due to differences in mineral content.

The first problem arises, therefore, from trying to arrange that comparable volumes are analyzed when comparing two different points in a specimen.

Soft Tissues

Reliable methods and apparatus for the preparation of thin, plane-parallel sections of soft tissues are generally available. These sections can be mounted on supporting blocks of a low atomic number material (e.g. carbon, which is cheap and safe to handle), or on grids, for examination in the microanalyzer. Since sections can be prepared which are thinner than the depth in which X-ray production is excited, it

should be possible to obtain reliable data in the form of mass per unit volume. The limiting factors will be the degree of accuracy to which the section thickness is known, and the freedom from distortion in the tissue at the moment at which the section is cut. Dehydration shrinkage should be limited to occurrence in the thickness of the section if it is mounted rapidly onto a supporting block.

Thin sections of soft tissues can be cut in the fresh-frozen state on a freezing microtome to avoid any contamination from fixatives. The knife material can be chosen so that there will be no confusion between fragments derived from the knife and the material of interest. For example, we used a glass knife to cut soft tissue in which we were looking for corrosion products (Fe, Ni, Cr) from a metallic implant.

Certain tissue components may be present in very small amounts or may have nearly the same elemental composition as the rest of the tissue. In either case the component could not normally be resolved by this method. However, in the first case it may be possible to concentrate the component without altering its distribution by drying down a thick section, if a considerable loss of distance resolution can be accepted. Lever and Duncumb (1961) boosted the FeK emission from rat duodenal epithelium in this manner. In the second case, where a component does not differ significantly in atomic composition from its surroundings, it may be possible to selectively attach other atoms to this component. Cosslett and Switsur (this Symposium) mention some preliminary experiments with this technique. In the study mentioned previously, Lever and Duncumb used the Prussian blue reaction to increase the iron content in some of their specimens. This sort of treatment is justifiable if it is known that the reaction used is quantitative.

Hard Tissues

Specimen Surface

The inhomogeneity of the specimens leads to difficulties in obtaining a flat surface finish. Surface irregularities affect the number of X rays reaching the spectrometer. Differences in the hardness of the specimen surface, and hence differences in the rate of abrasion during polishing procedures, may be due to differences in mineral content or to differences in the crystallographic orientation of this mineral content. Thus, the surface irregularities produced during polishing may be related to the structure of the tissue and the differences in elemental distribution which are of direct interest. Since denser regions in a mineralized tissue will tend to be harder and more abrasion-resistant, they will finally occupy a more prominent position. This effect leads, therefore, to an accentuation of the real differences in mineral content (Fig. 1). The problem is

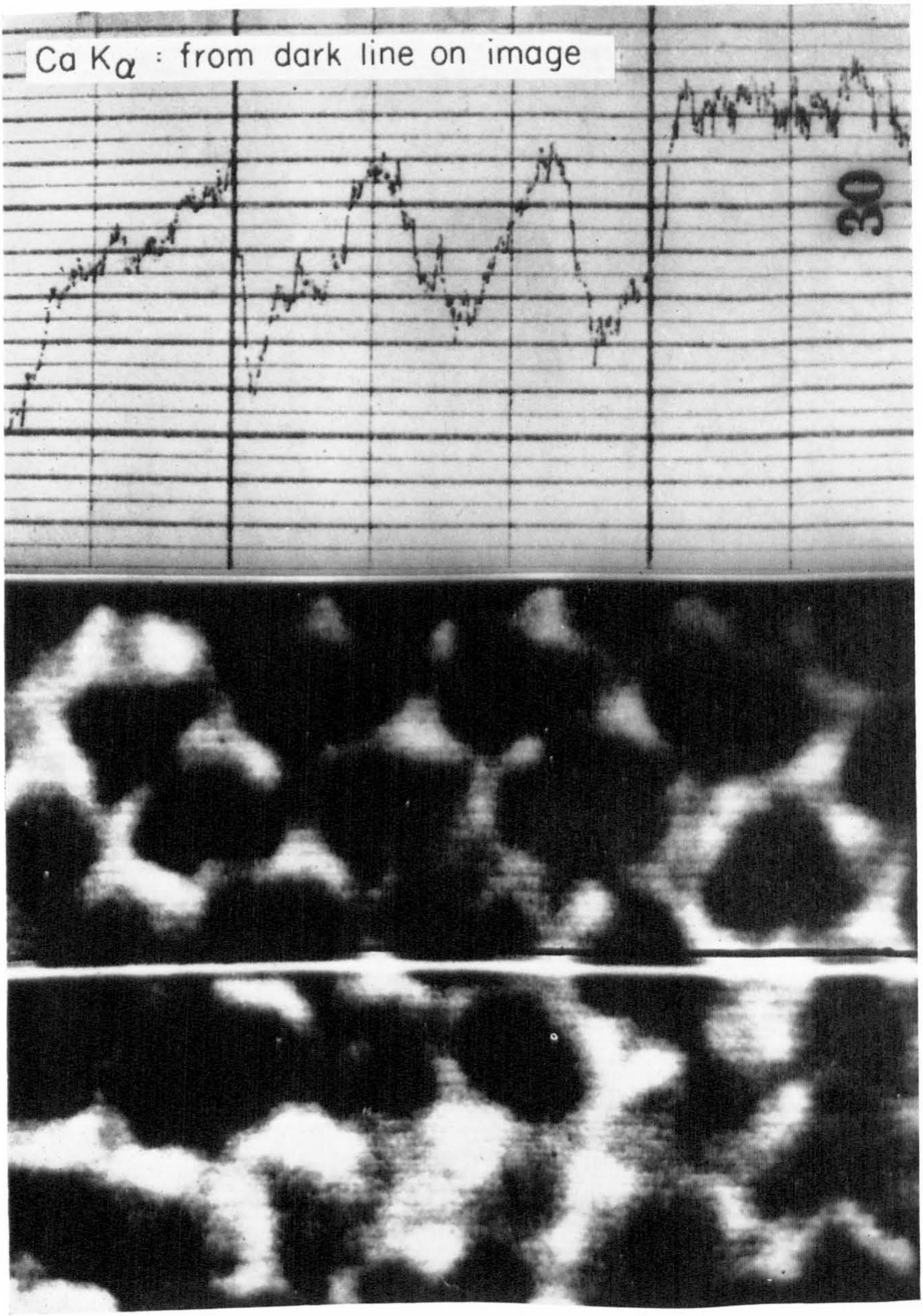

FIG. 1. Electron image of surface of transversely sectioned prisms of carious human enamel. Ca $K\alpha$ line trace taken along line marked on the image; count varies from approximately 65 to 85% of the value obtained from pure hydroxyapatite under the same conditions. This variation is surprisingly small.

greatest in the case of soft-hard tissue junction regions. The soft tissue components will not be retained in place during a sawing or grinding and polishing process, unless the whole block of tissue is permeated with a material which hardens *in situ* (e.g., methyl methacrylate). A representative surface cannot be displayed by simple microtome techniques because too much shattering and compression occurs (Figs. 3 and 4).

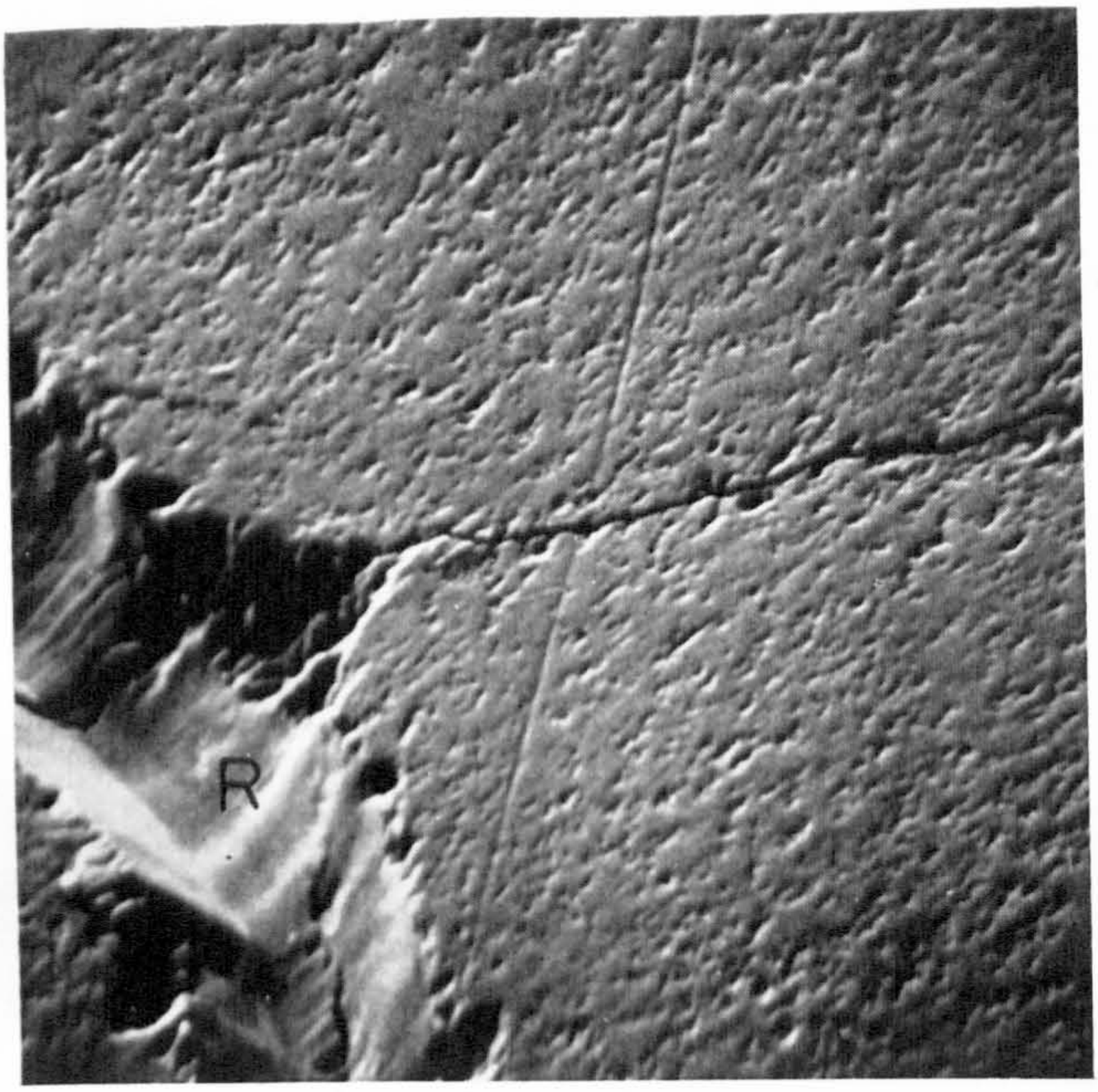

FIG. 2. Scanning electron micrograph of "polished" human enamel. R = Reference scratch. (Courtesy of Dr. A. D. G. Stewart.)

Therefore our hard-tissue specimens, to date, have been the carefully polished surfaces of plastic-embedded material (Fig. 2).

In a number of hard tissues there are cavities which are occupied by cells or cell processes in life, and these may act as traps for electrons which would normally have been backscattered, resulting in a local increase in X-ray production. This would be so if the cavity were filled with a low atomic number material such as plastic, but such a cavity may become filled with polishing debris if the material is not embedded.

It has been suggested that the ordinary polishing procedures used in preparing tooth section surfaces may leave a damaged surface layer of the same depth as the layer analyzed by the electron probe (Boyde and Stewart, 1962).

The existence of such a damaged layer is significant because it means that a lateral shift of surface material can occur, and this would detract from the resolution. In considering how the damaged layer forms on dental enamel, it is of significance that J. C. Elliott (personal communication) has prepared a hard, transparent disc of hydroxyapatite by pressing together small synthetic crystals of this material in the dry

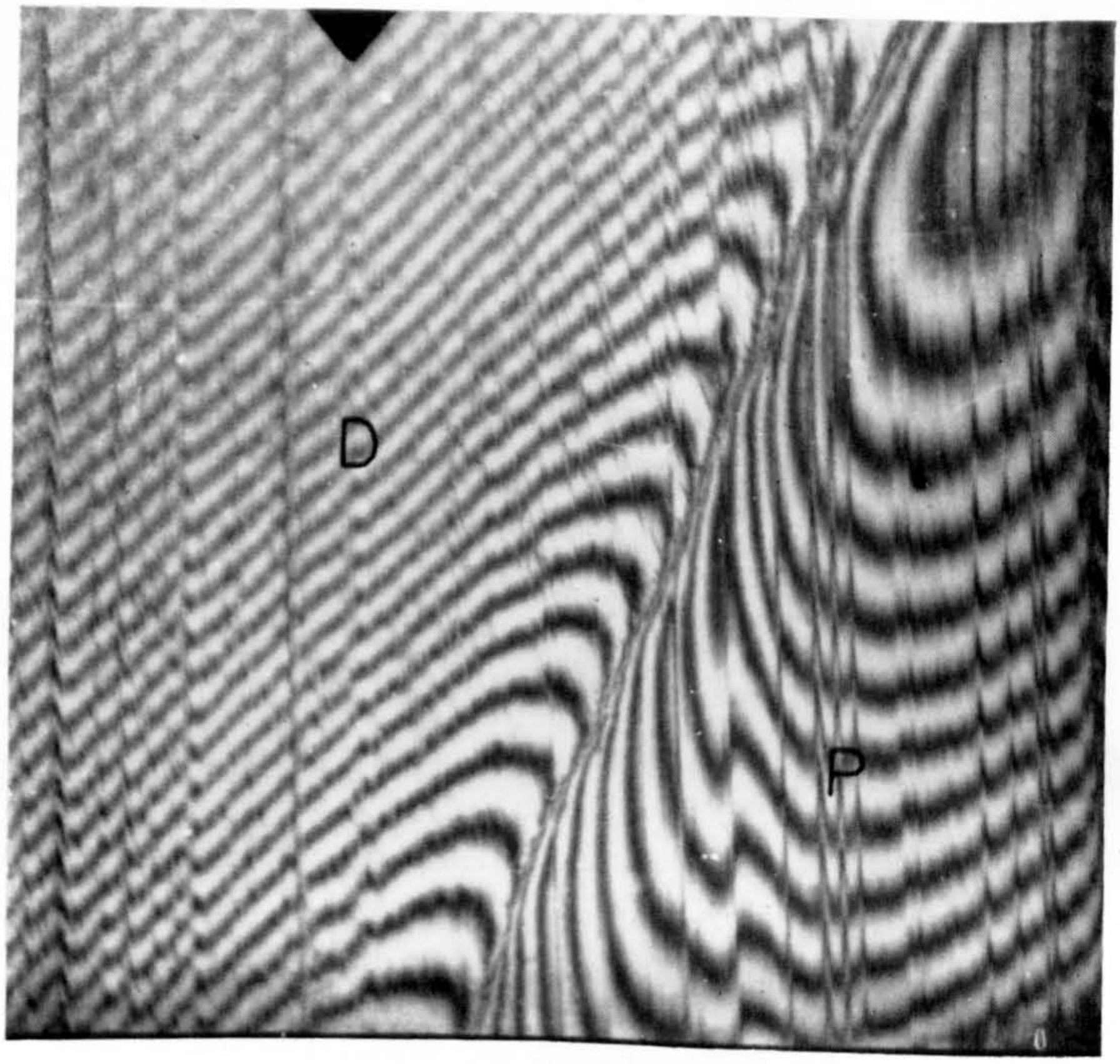

FIG. 3. Photomicrograph of human pulp-dentine junction; methacrylate-embedded material cut with a diamond knife. Cut surface of the block viewed with interference microscope. Interference fringes form contours which, by their displacement, show a large difference in height of the surface between dentine (D) and pulp (P) caused by compression during the cutting procedure.

condition in the same way that KBr discs are prepared for infrared spectroscopy.

Thus it is conceivable that a degree of repacking of the abraded material may occur. Another possibility is that plastic flow may occur under locally high pressure during the polishing process. It does not seem to be widely appreciated that plastic deformation can occur in brittle nonmetallic crystalline substances. For this reason we append some references to plastic flow: in sapphire, rock salt, and limestone,

Bridgman (1952); in diamond, Seal (1956); and in dental enamel, Berlin (1959) and Schmidt (1961).

It is possible that acceptably flat, undamaged specimen surfaces may be produced by a cutting technique using high-velocity gas-propelled abrasive particles at a low angle of incidence to the final surface. In this case there could be no high-pressure effect, and no possibility of a "re-

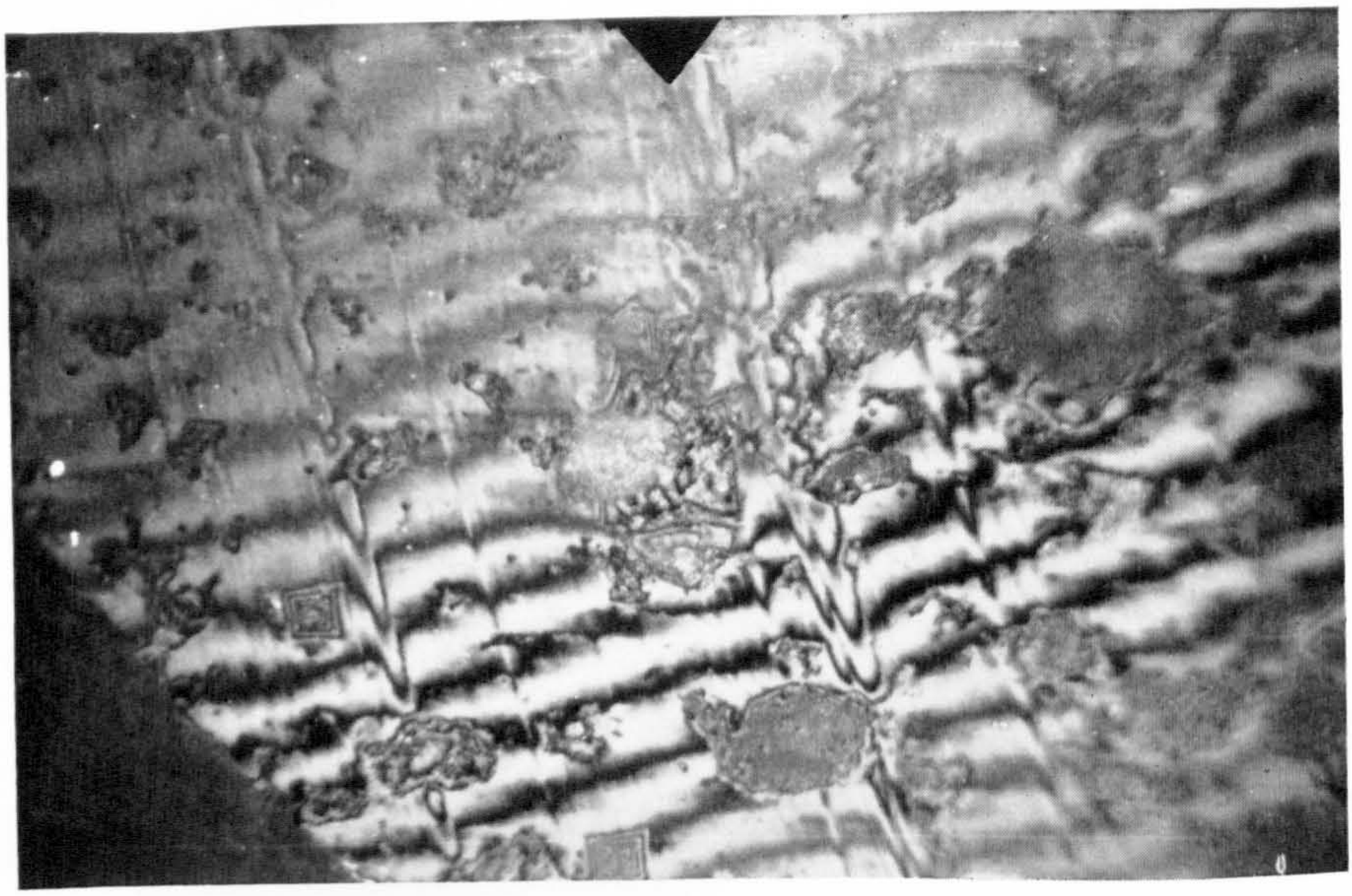

FIG. 4. Photomicrograph of surface of human enamel cut with diamond knife and viewed with a surface interference microscope. Large fragments have been torn from the surface during cutting, and ridges are also present. Therefore, cutting or scraping techniques do not leave a satisfactory finish. The square areas are microhardness-testing indentation marks.

constitution" of the eroded material. Some preliminary tests using the S. S. White Industrial Airbrasive unit with alumina particles have given quite encouraging results. As a final finishing technique, the use of a cathode sputtering by inert gas ions at low angles of incidence may prove useful.

MASS PER UNIT VOLUME DATA FROM HARD TISSUES

The problem of obtaining mass per unit volume data for an element from hard tissues (caused by the density differences which exist here) can be approached in two general ways. Either (1) the specimen can be prepared as a thin plane-parallel section of known thickness, thinner

than the depth in which X-ray production is excited, or (2) one phase of the specimen can be substituted in order to bring the specimen to a uniform density. We have not had much success with either approach.

(1) We examined sections ($\sim 1\,\mu$) cut with a diamond knife on an ultramicrotome, but it is our impression that the X-ray contrast from these specimens was derived chiefly from variations in their thickness. Under the best conditions, sections of dental enamel as thick as $4\,\mu$ can be prepared by ordinary grinding and polishing techniques. These are, of course, thicker than the depth in which X-ray production is excited, and the existence of a damaged surface layer should discourage their use. Sections of dentine as thick as $1\,\mu$ have been prepared and these would be acceptable if the variations in thickness were known.

(2) If an element of interest were known to be a part of the organic matrix of a mineralized tissue, it would, of course, be easy to remove the mineral component by acid decalcification, and to substitute it with a plastic embedding material of the same density as the organic matrix.

We removed the organic phase from tooth fragments by refluxing them in ethylenediamine. We then filled up the spaces so formed with a salt of the same density as "bone salt." This was done by immersing the treated section in a solution of sodium tungstate, which was allowed to crystallize out very slowly. A surface was then prepared through the center of the tooth fragment. Unfortunately, tungsten has a much higher backscattering coefficient for electrons than either calcium or phosphorus, so that the enhanced differences in calcium concentration which were observed may have been partly due to this. However, it should still prove possible to select a substituting medium with desirable density properties.

Conducting Surface Layer

All biological specimens are nonconductors of electricity when dehydrated. We have used evaporated films of copper ($\sim$50 A) occasionally, and aluminum ($\sim$200 A) routinely, to form conducting layers on the surfaces of our specimens. Aluminum evaporated onto highly mineralized tissues always forms an intact silvery film, but forms a black film on unembedded bone or dentine. Although the exact nature of this film is not known, it may not be acceptable as a conducting film since Holland (1956) mentions that black films under other circumstances have high resistivities. It is probable that the black film results from a large out-gassing from the more highly hydrated tissues. Deliberate attempts to form black films are conducted at pressures of the order of 10^{-1} mm Hg.

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A Consideration of Some Design Features of a Scanning Microanalyzer for Biological Applications

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There have been only a limited number of biological applications of the electron-probe microanalyzer. Our experience with the instrument constructed at the Cavendish Laboratory has led us to the conclusion that useful information could be obtained with this technique from most biological specimens without too much thermal damage. There is certainly a wide scope for the use of this method in biology, especially if the localized quantitative aspect could be exploited, since the number of absolute methods in this field is limited.

We have examined a variety of heat-susceptible materials, including hard and soft tissues, easily volatile or fusible materials of poor thermal conductivity such as semiconductors, and easily decomposable substances such as the pigments in ancient paint films. All these materials are affected by heat to a larger extent than metals when subjected to bombardment by the electron beam. They have a much smaller thermal conductivity and are subject to a considerable temperature rise if the normal probe current of about 1 μa is used. In addition, they are subject to electrostatic charging to some extent even when covered by a metallic conducting film. These factors place practical limits of 1 to 5×10^{-8} amps on the probe current at 20 kv accelerating voltage.

The susceptibility of biological tissues to temperature changes thus creates a demand for an instrument which can utilize the relatively small number of X rays produced by a limited beam current in a much more efficient manner.

Difficulties in machining flat, undamaged specimen surfaces are inherent in the inhomogeneity of biological material at the microscale. Since means of producing biological specimens with plane surfaces have not been perfected it is necessary to use as high a take-off angle as possible for the X rays, say 45° to 60°, in order to reduce self-absorption of the X rays, which is very marked with wavelengths longer than the approximately 3.5 A which are under consideration here.

The decomposition of the specimen can be delayed by scanning the beam over an area of the sample. However, even with a reduced probe current of 5×10^{-8} amps some disturbing variations in the X-ray output with time have been measured. This variation does not appear to be related to the build-up of contamination on the surface of the specimen. The continuous conducting film provided to prevent charging by the incident beam does not help significantly to reduce the thermal difficulty. When the specimen decomposes, this film is disrupted and electrostatic charging occurs. To help combat these peculiarities of biological and other heat-sensitive specimens, the following design features are suggested for a microanalyzer intended for their investigation.

Since the time available for the analysis is limited, it is necessary to obtain as much information as possible by using several analytical channels simultaneously. The total X-ray intensity is low so that X rays must be collected through larger solid angles. The density of the specimens is low and this enables the electrons to diffuse and produce X rays throughout a reasonably large volume, the resolution thus being limited to about 2 μ unless a low excitation voltage is used.

With these limitations in mind, the design of the probe-forming lens can be changed from the current vogue of short focal length and a small probe diameter with high incident current density. Instead, the lens would have a long working distance of the order of 5 or more centimeters, and would deliver a maximum of about 5×10^{-8} amps at 20 kv into a probe of 1 μ diameter. The probe would be scanned over the specimen surface in a raster of up to 1 mm side, and there would be facilities for displaying images formed by electrons as well as X rays. The intensity of X rays recorded by the detectors would vary with the irregularities of the specimen surface and depend strongly on the direction of the detector. For example, a projection from the surface of bone only a micron in height would reduce the intensity of calcium X rays received at a spectrometer at 20° to the surface by over 10%. To reduce error from this type of effect two detectors situated at 180° from each other would be set to detect the same radiation to provide an internal control. Adding together the information received from spectrometer pairs grouped at 180° from each other would also help to reduce errors due to the relative movement between the X-ray source, i.e., the probe position, and the spectrometer.

The elements contained in the specimen would in most cases be known, and only their distribution or quantitative measurement would be required. Hence the detectors may be set to receive a definite radiation from the specimen. The information must be recorded before decomposition can affect the results and so, in a typical case, three sets

detector pairs would be arranged to receive the information simultaneously. Because of the long working distance of the final lens, it would be possible to group the detectors very close to the specimen and thus utilize a larger proportion of the emitted X radiation than is done with present instruments. This would help to compensate for the lower intensity generated by the use of the small currents that can safely be employed. The detectors would probably be proportional counters followed by a matrix system for separating pulse-height distributions (Dolby, 1959; Dolby and Cosslett, 1960) or simple crystal spectrometers that would be preset for the required element. A series of plug-in spectrometers would be available, together with a single scanning spectrometer which might be useful on some occasions. Constant monitoring of probe and/or specimen current would be desirable.

It would be useful if the specimen could be a large one mounted on a glass microscope slide for convenient transfer to other apparatus, and if it could be viewed during the electron bombardment through an optical microscope. This feature has been incorporated in Long's instruments (Long, 1959). It would be capable of being traversed manually or mechanically in orthogonal directions as well as being rotated about the point of impact of the undeflected electron beam. A series of standard samples of elements would be rapidly interchangeable with the specimen.

Display and the recording of information would follow conventional lines with versatile time bases for probe positioning, scanning, slow-line and slow-field scanning.

Since the cost of such an instrument would be high it would gain entrance to many medical or biological laboratories only if it could be made multipurpose, and it would be useful in this respect if the facility to count, record, and use X rays in the forward direction were incorporated so that it could be used for point-projection microscopy, microdiffraction, and fluorescence analysis.

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INVESTIGATION OF THE EROSION OF TOOTH SECTIONS WITH AN ARGON ION BEAM

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The specimens were tooth sections stuck to brass or aluminium backing plates with Araldite or Eastman 910 adhesives. The exposed surfaces were polished on metallurgical polishing papers down to grade 4/0, cleaned by stripping off formvar replicas or washing with acetone, and finally covered with a layer of evaporated aluminium approximately 300Å thick. The final thickness of the sections was 50–100μ. The specimens were bombarded with 5 kV argon ions at 45° incidence in the specimen chamber of a scanning electron microscope (1). It was not necessary to move the specimens between the operations of etching and viewing. The scanning electron microscope accelerating voltage was 12–15 kV. The pictures were formed by collecting only the high energy reflected electrons. No trouble was experienced due to charging by either the ion or electron beams.

Nickel dust particles were placed on the specimens and shielded part of their surfaces from the effect of ion bombardment. It was thus possible to estimate the thickness of material which had been removed from each part of the specimen, by measuring stereo pair micrographs of the nearest shielded area (Fig. 1). The maximum rate of removal of material from the surface was 1μ per minute. Sequences of stereo pair scanning of electron micrographs were prepared showing the changes in topography with ion dosage. The profile produced depended only on the thickness removed and was not influenced by the rate at which the surface had been eroded. After removal from the scanning microscope, replicas were prepared by various techniques and they were examined in a Siemens Elmiskop I. The ion-eroded surfaces were hard; many replicas were taken and no crystallites pulled away from the surfaces. The specimens were also examined by light microscopical techniques before preparation and after ion erosion.

We assume that, apart from a limited degree of cracking, thermal effects were negligible, because; 1) the temperature, as measured by a thermocouple on the back of the specimen mounting plate, was always less than 23°C; 2) the estimated temperature difference across a 100 μ section in the region of the maximum ion current density was less than 100°C; 3) examination of ultrathin sections cut transverse to the original specimen surface in the Siemens Elmiskop I showed no alteration in the surface layers, which would have been expected had the specimen been overheated. The specimen surfaces were eroded differentially. Dentine was eroded away faster than enamel, intertubular dentine faster than peritubular dentine (Fig. 1) and tuft enamel faster than the surrounding normal enamel (Fig. 2). We think that these differences in sputtering rate are principally related to differences in mineralisation, since the more rapidly eroded material is less highly mineralised in each case.

Differences in the degree of mineralisation throughout the bulk of enamel are small, but differential etching still occurred, thereby revealing the rod structure. This suggests that there is a variation of sputtering coefficient with crystallographic orientation of the hydroxyapatite crystallites, similar to that which has been observed with metallic crystals, since differences in hydroxyapatite crystallite orientation are the most prominent feature of enamel structure. The method may, therefore, have some application in the study of differences in crystallite orientation at a surface and we will give two examples of this use. In many areas the enamel surface was initially eroded uniformly and differential etching did not occur until a few microns of the polished surface had been removed (Fig. 2). This would seem to indicate that the surface polishing procedure that we employed had produced a damaged amorphous layer. There was also a very limited differential etching in the outermost 10–20μ layer of the enamel (Fig. 3). This suggests that the crystallites in this surface zone of the tooth have predominantly one orientation.

1) A.D.G. Stewart - this conference.

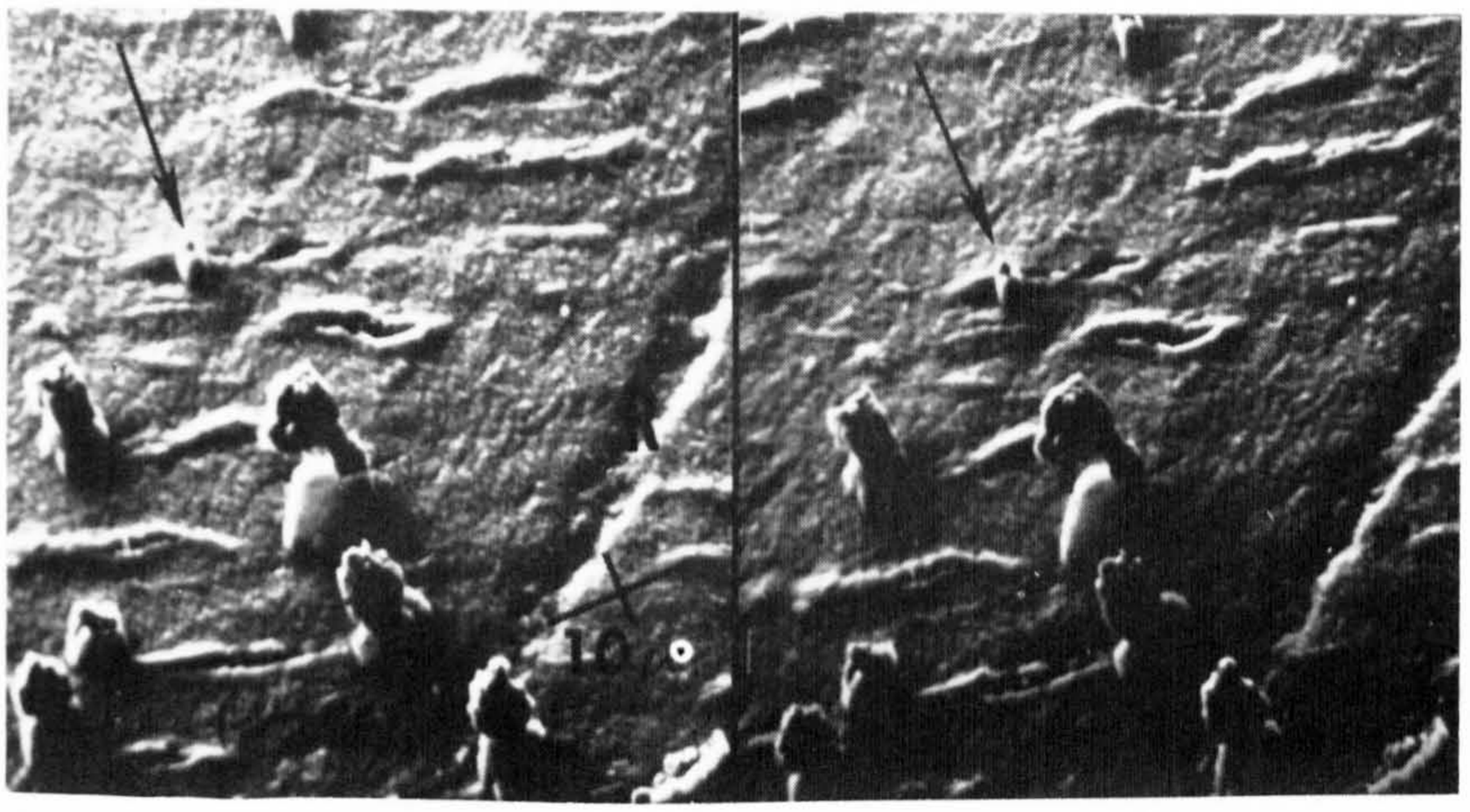

Fig. 1. Scanning electron micrograph stereo-pair of ion eroded surface of longitudinal section of human dentine. The difference in height between the original surface preserved under the shielding nickel particles and the eroded surface = thickness of material removed. The peritubular dentine can be seen to stand above the intertubular dentine. Arrow shows ion beam direction. R = Reference Scratch.

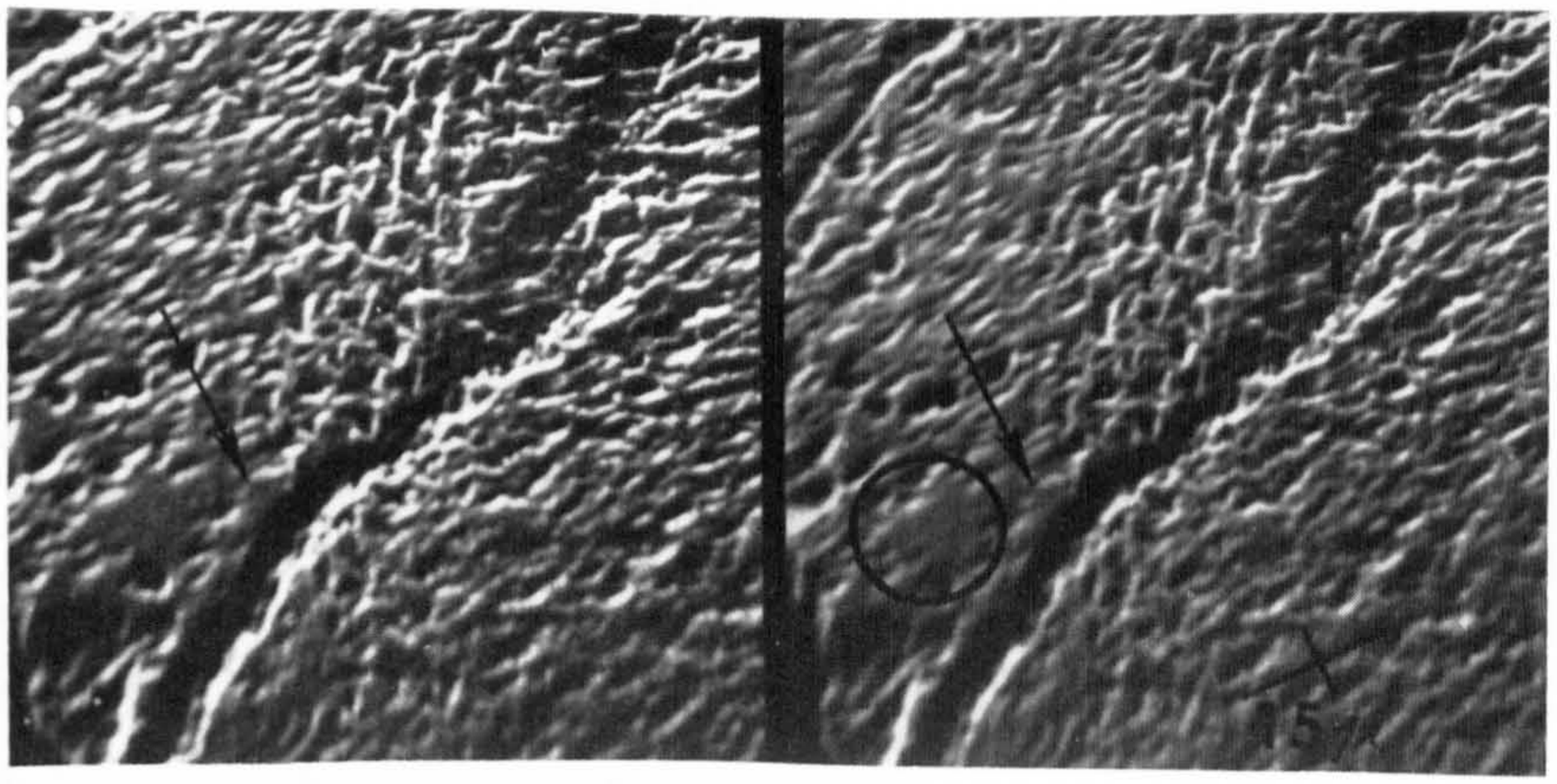

Fig. 2. Scanning electron micrograph stereo-pair of ion eroded surface of oblique section of human enamel. T = tuft enamel eroded deeper than surrounding enamel. Circle on left hand picture outlines an area in which differential etching had not yet commenced after some 5 μ of the original surface had been eroded away. Arrow shows direction of ion beam.

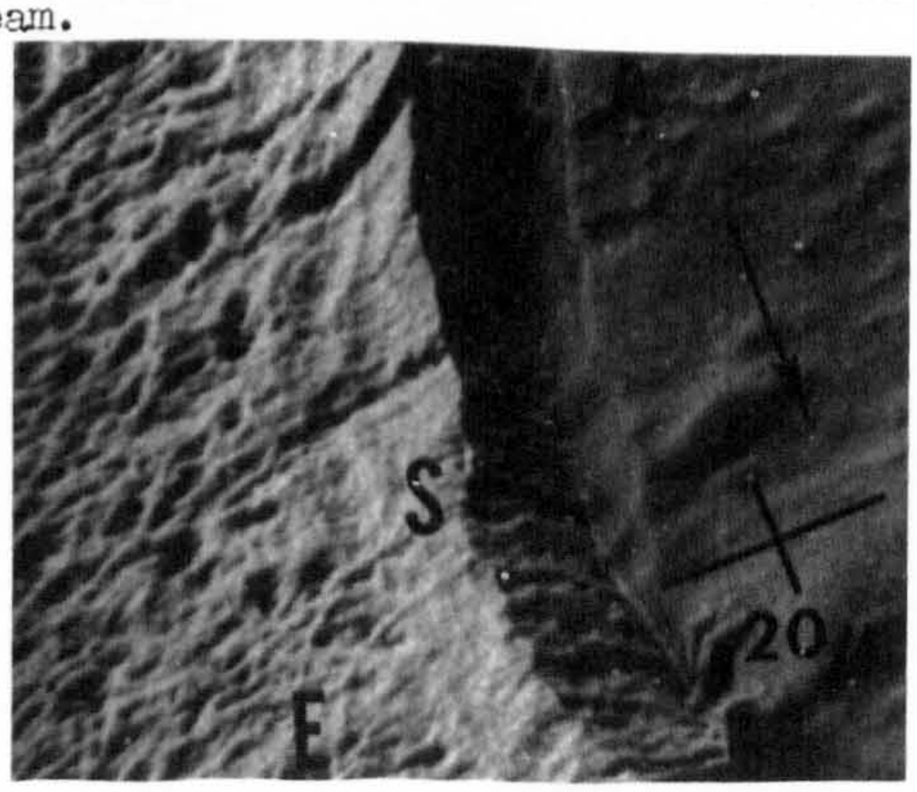

Fig. 3. Scanning electron micrograph of ion eroded surface of transverse section of Macropus enamel. The rod structure can be visualised throughout the enamel, except in the surface zone - the true surface of the tooth. Arrow shows ion beam direction. A = Araldite. E = Enamel. S = Enamel 'surface zone'.

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AN ASSESSMENT OF TWO NEW PHYSICAL METHODS APPLIED TO THE STUDY OF DENTAL TISSUES

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Abstract—X-ray emission micro-analysis and ion-beam erosion etching have been applied to the study of dental tissues. The former gives information about the elemental composition of the surface layer of the specimen, derived from the X-ray emission spectrum excited by direct electron bombardment. Both methods reveal differences in composition (e.g. degree of mineralization) and the second may also reveal differences in structure (e.g. crystallographic orientation of hydroxyapatite crystallites at the specimen surface) though it can neither distinguish between these differences nor derive absolute information about crystallite orientation.

Résumé—Les deux méthodes envisagées dans ce travail sont d'une part la micro-analyse par émission de rayons X et d'autre part la gravure par érosion dans un flux délié d'ions accélérés.

La première méthode renseigne sur la composition élémentaire de la surface de l'échantillon par la spectrographie des rayons X émis par cette surface sous bombardement par un faisceau d'électrons.

Les deux méthodes révèlent les différences locales de la composition superficielle de l'échantillon, notamment quant à la minéralisation.

La seconde méthode peut encore révéler des différences structurales, comme l'orientation cristallographique de l'hydroxyapatite des cristallites affleurant la surface de l'échantillon. Elle ne permet toutefois pas d'en déduire l'orientation du cristallite lui-même.

Zusammenfassung—Die beiden in dieser Arbeit vorgeschlagenen Methoden sind einerseits die Mikro-Analyse durch Röntgenstrahlenaussendung und andererseits die Erosionsgravur durch einen feinen Strahl beschleunigter Ionen.

Das erste dieser beiden Verfahren gibt über die elementare Zusammensetzung der Oberfläche des zu untersuchenden Musters Auskunft durch Spektroanalyse der von der Oberfläche ausgesandten Röntgenstrahlen infolge Elektronenbeschusses.

Das zweite Verfahren kann strukturelle Unterschiede enthüllen wie die Kristallorientierung des Hydroxylapatites der Kristallite, die an die Musteroberfläche reichen. Es erlaubt jedoch keine Rückschlüsse bezüglich der Orientierung der Kristallite.

Die beiden Verfahren geben über die lokalen Unterschiede in der Oberflächenzusammensetzung des Musters Auskunft, speziell hinsichtlich der Mineralisation.

IN previous papers (BOYDE, SWITSUR and FEARNHEAD, 1961; BOYDE and STEWART, 1962) we have described the application of two physical methods which we believe are new in the field of study of dental tissues, but which have been fairly extensively applied in metallurgy. These methods are X-ray emission micro-analysis and ionic erosion etching. The purpose of this paper is to re-assess the value of these methods

in their current state of development and to mention the chief lines along which improvements must be made.

SCANNING ELECTRON PROBE X-RAY MICRO-ANALYSIS

The phenomena of X-ray diffraction and absorption have been widely employed by dental researchers, and some use has been made of X-ray fluorescence. Little attention has been given, however, to the direct excitation of X-rays by an electron beam from a specimen surface, in spite of the fact that the information available from the spectroscopy of these X-rays is relatively easily interpreted. Furthermore, the X-ray emission which can be excited from very small, accurately localized areas can be measured using the technique of scanning electron probe X-ray micro-analysis (DUNCUMB and COSSLETT, 1957). This method is, in fact, potentially the most accurate for obtaining data about elemental distribution on the micro-scale. Present limitations of this technique are imposed by both the methods for specimen preparation and the fact that the prototype instruments were designed for metallurgical applications.

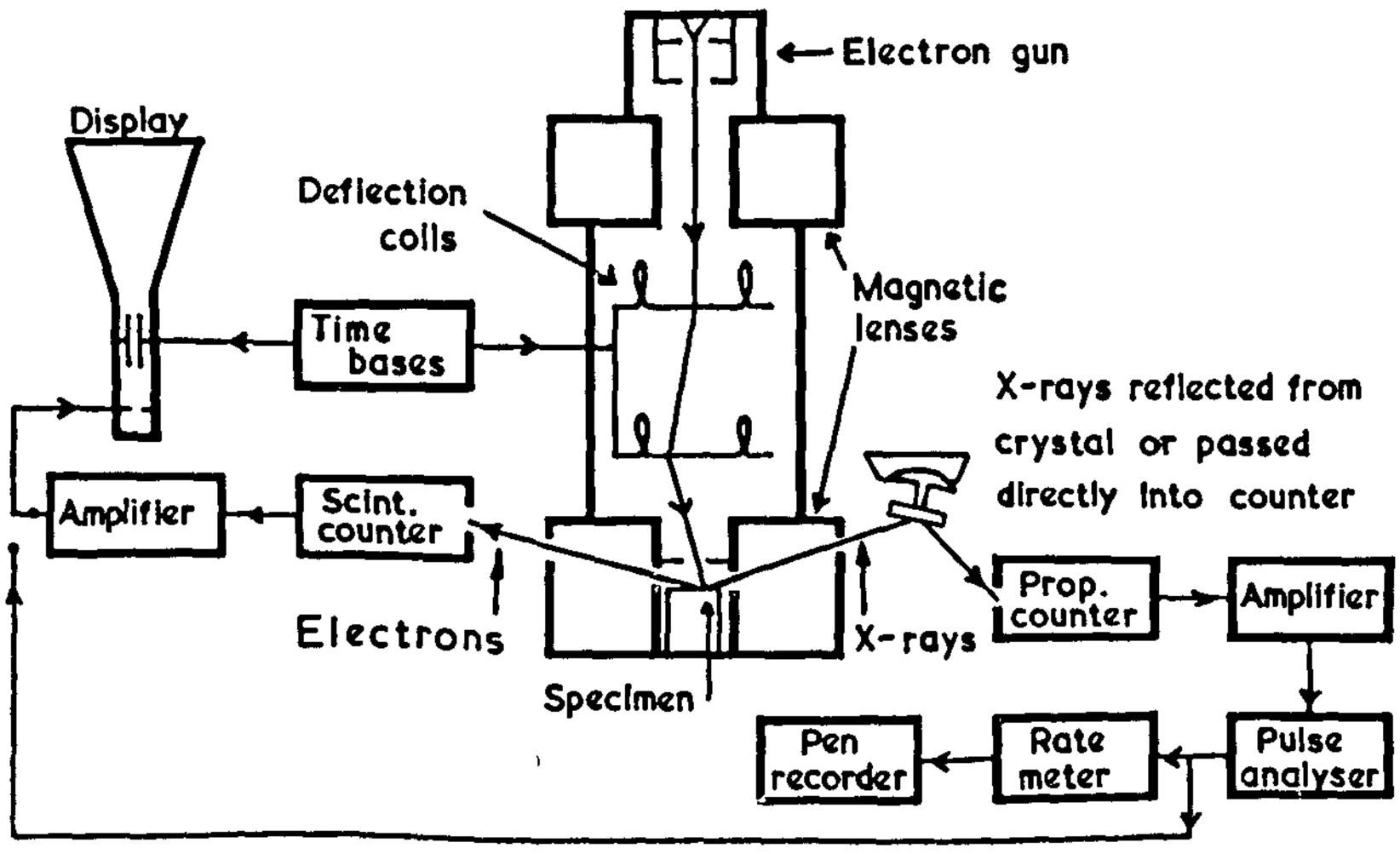

FIG. 1. Diagram of the scanning electron probe X-ray micro-analyser.

The scanning electron probe X-ray micro-analyser (Fig. 1) utilizes the X-ray emission spectrum resulting from the bombardment of elements by an electron beam. Qualitative and quantitative data can be obtained, which can be related to the topography of the surface of the specimen. Thus it is possible to identify an element and obtain an accurate estimation of the quantity of this element in the surface of the sample.

As an example of the sort of results obtainable from this instrument, we have included Fig. 2. This shows a line trace recorded in Ca Kα radiation across some transversely sectioned carious enamel prisms. Differences in the intensity of emission can be related to a given line of the scanning electron image of the surface.

This enables us to relate the points of highest intensity on the recording to the "interrod" regions (in this case) and also to derive estimates of the calcium concentration at different points. The contrast in the electron image is determined, partly by the physical form of the surface and partly by its elementary composition

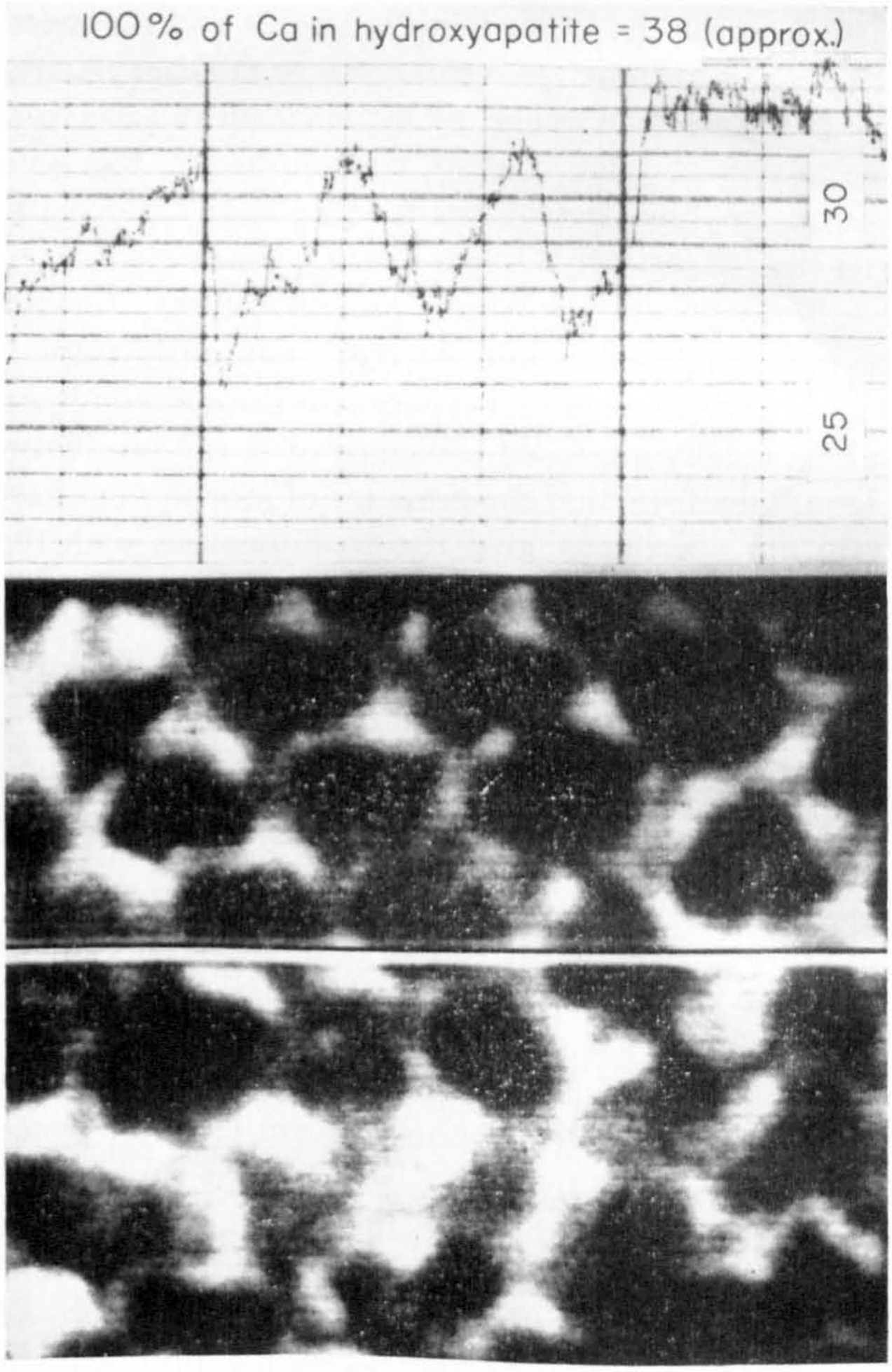

FIG. 2. Scanning electron image of human premolar carious fissure enamel with line trace in Ca Kα radiation recorded whilst moving probe slowly along the line marked on the image. This micrograph was prepared on the Cavendish Laboratory Instrument.

(Atomic number contrast). Examination of the Ca Kα recording from this specimen shows less apparent variation in the calcium concentration across these prisms than might have been expected, since there is such marked contrast in the electron image. This specimen, therefore, serves well to demonstrate the main difficulties of interpretation with this type of material. It is apparent that the centre of the enamel prisms have been selectively attacked by the carious process in this region. This

introduces the distinct possibility that, owing to the relative softness of these regions, they would be worn away preferentially during the polishing procedure, leaving the "interrod" region standing proud. Thus the inherently high atomic number contrast in the electron image, due to the high calcium concentration in the interrod regions, would be accentuated by the shadowing effect due to the difference in height between the lower lying rod cores and the more elevated interrod regions. Although the above provides a reasonable explanation of the high contrast available in the electron image, an explanation still has to be found for the limited difference in the recorded calcium concentration. The volume penetrated in the specimen surface by the electron beam depends on the density of the material in that region, and thus in regions of lower density, the electron beam will penetrate further and excite X-ray production from a greater volume. Furthermore, one may surmise that the electron beam would be more efficiently trapped by the open, pitted ends of the rods, which would act as cages for the electron beam and that there would therefore be a locally increased efficiency of X-ray production from the rod cores. It can be seen, therefore, that the presence of morphological holes and differences in density in our specimens give rise to difficulties with the interpretation of the quantitative data, since one cannot be sure that equal volumes of the specimen surface are being compared.

In general principle, this problem might be tackled in two different ways. Firstly, one might attempt to ensure that the whole thickness of the specimen is analysed by allowing the electron beam to pass right through the specimen. This implies the use of a thin ($\sim 2\mu$) plane parallel section as the specimen. Secondly, one might attempt to ensure that a surface layer of uniform depth is analysed, by bringing the specimen to a uniform density. In the case of hard tissues, where we are interested in the distribution of hydroxy-apatite, the organic material could be removed and replaced with a salt of the same density as hydroxy-apatite, but not containing calcium or phosphorus. We have used sodium tungstate for this purpose, which was an unfortunate choice because tungsten has such a high back-scattering coefficient for electrons. This results in non-uniform electron penetration in areas containing different amounts of tungstate. However, it should be possible to find a more suitable substitute, when this principle might be tested more fully.

The susceptibility of all biological tissues to large temperature changes imposes more stringent requirements upon the design features of a micro-analyser intended for use in this field than on a similar instrument for metallurgical applications. Thus the incident electron beam must be limited to not more than about 10^{-8} A when using a 20 kV accelerating potential compared with an acceptable 10^{-6} A on a metal specimen. The relatively small number of X-rays which can be produced from biological material have to be utilized in as efficient a manner as possible, and the information must be obtained as rapidly as possible consistent with statistical accuracy by analysing for several elements simultaneously (SWITSUR and BOYDE, 1962). As far as the authors are aware, a suitable microanalyser for biological material does not yet exist, whereas the problems requiring such an instrument certainly do. It may be, therefore, that our time would be best spent, whilst we await

the construction of a better instrument, in solving the extremely difficult specimen preparation problems. This leads us to a consideration of the second method discussed in this paper, which has a direct bearing on the improvement of specimen preparation techniques.

THE EXAMINATION OF SURFACES UNDER IONIC BOMBARDMENT

Stewart, at the Engineering Laboratory at Cambridge, has recently been investigating the characteristics of metal surfaces under ionic bombardment in a scanning electron miscroscope. It occurred to us that ionic bombardment might be suitably applied to the preparation of the polished surfaces of sections of dental tissues, prior to the use of other methods of study. An investigation was, therefore, initiated and a preliminary report has been prepared (BOYDE and STEWART, 1962). In this series of experiments we bombarded the polished surfaces of tooth sections, with a 5kV argon ion beam at 45° incidence in a vacuum of 10^{-6} mm Hg, in the scanning electron microscope with an attached ion source (Fig. 3) developed by Stewart for his investigation.

We found that erosion of the surfaces under bombardment took place; that dentine was eroded more rapidly than enamel (Fig. 4), intertubular dentine more rapidly than peritubular dentine, and that "tuft" enamel was eroded away more rapidly than the surrounding normal enamel. We concluded that the above differences in the rate of removal of surface material under ion bombardment (sputtering rate) were most probably related to differences in density. We also observed that differential etching occurred within the enamel, where differences in the degree of mineralization (if they exist) are certainly not as great. We concluded that the differential erosion that occurred in enamel was related to the differences in hydroxy-apatite crystallite orientation at the surface. It has been shown for other crystalline substances, that the rate of removal of material from the surface by a given ion beam (constant voltage and atomic number) at a given angle of incidence is strongly dependent on the angle that the crystal lattice makes with the surface.

It is probable that a great deal more information may be derived from the application of this technique, when the variation in the sputtering coefficient (atoms removed per ion) of hydroxy-apatite with the orientation of the crystal lattice, and the angle of incidence of the incoming ion beam, have been measured. In the meantime we may make some use of the technique even without this information. For instance, if we find that differential erosion does not occur at a surface, then we may infer that this surface is uniform in structure. However, we cannot deduce whether it is uniform in the sense of its presenting one crystal direction to the ion beam or whether it consists of many very small crystallites having a random orientation. We have, in fact, noted three regions in which differential erosion was limited. These are:

1. The true surface (10–20μ) zone of the enamel of the tooth shown in Fig. 5 was eroded differentially, to much less an extent than the rest of the enamel. This finding lends support to the idea that the crystallites in this surface zone of enamel

have predominantly one orientation and/or may indicate that this surface zone is uniformly mineralized.

2. A very limited degree of differential erosion occurred in a zone surrounding an enamel caries lesion shown in Fig. 6. This corresponds in position to Gustafson's

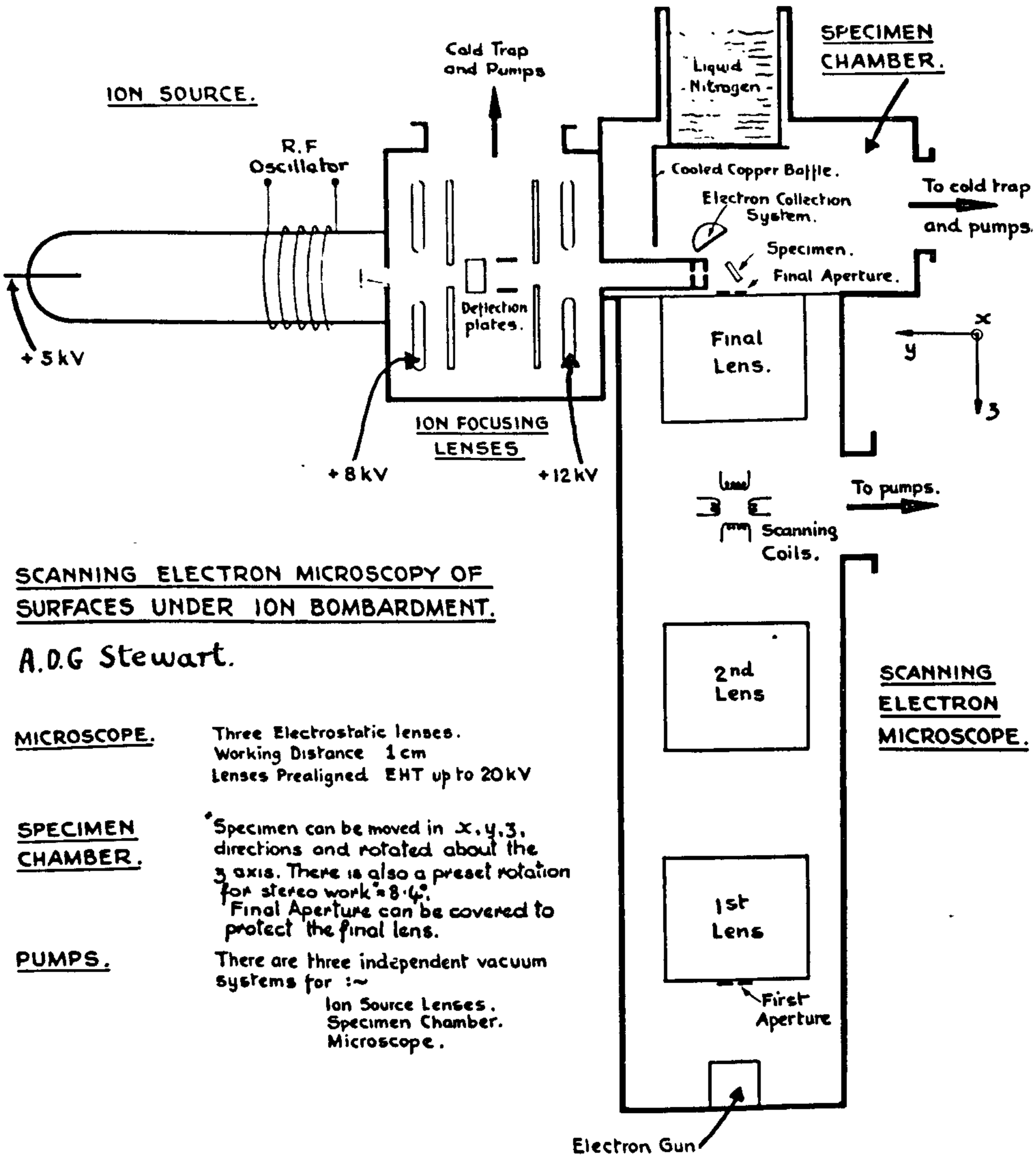

FIG. 3. Diagram of the scanning electron microscope with attached ion source.

(1957) zone 1, as determined from the polarized light control photomicrographs. This result would suggest a uniformity of some nature in this layer, which is not possessed by the enamel to either side of it.

3. In most cases, differential erosion of polished enamel surfaces did not commence until a few microns of the surface material had been eroded away. Such areas

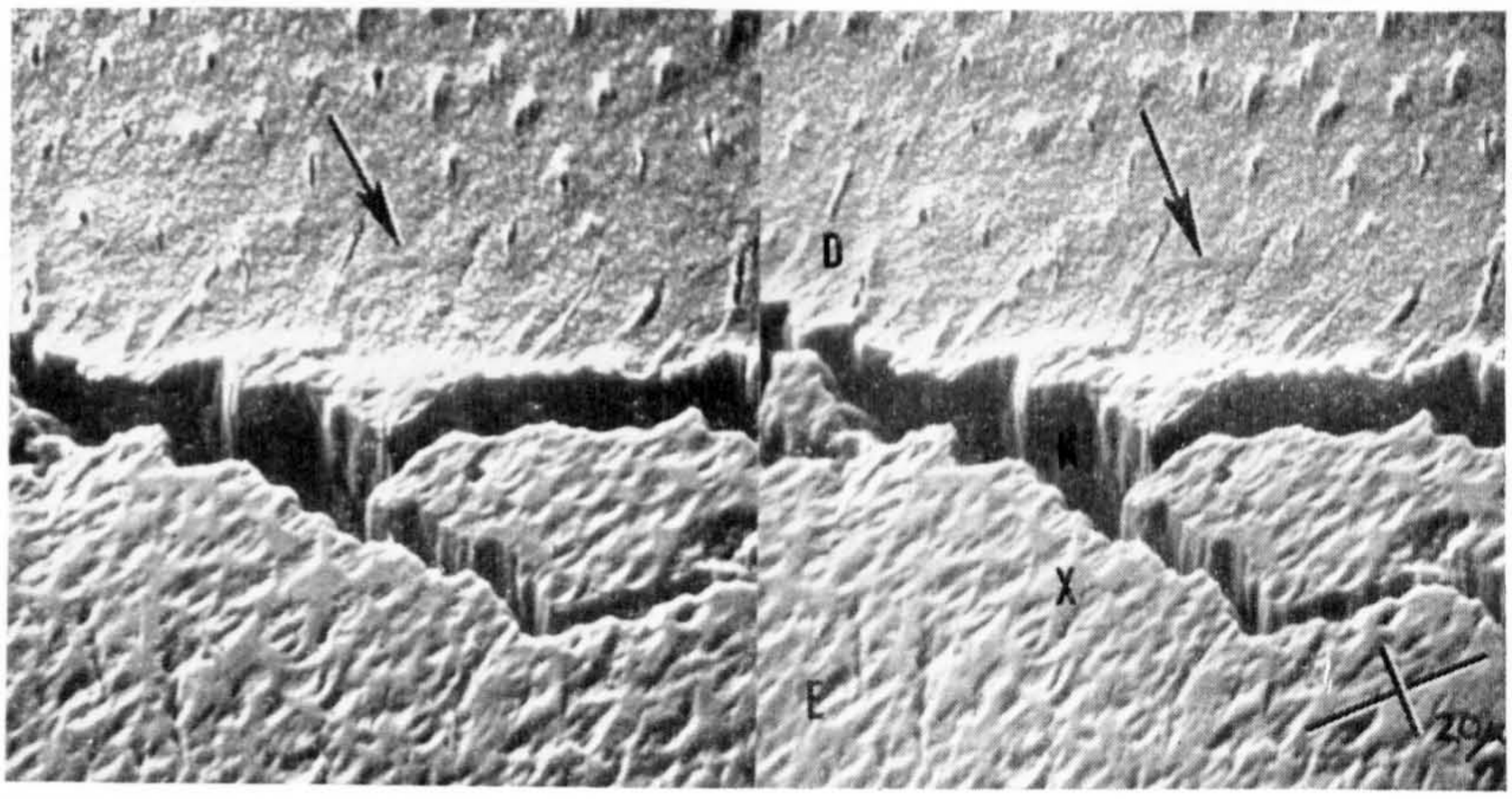

FIG. 4. Scanning electron micrograph stereo-pair of polished, argon ion-beam eroded surface of oblique section of human premolar. Enamel above—dentine below. D, dentine; E, enamel; K, crack; X, area in which differential erosion has not yet commenced.

N.B. In Figs. 4, 5 and 6 the arrows show the ion-beam direction. The specimen was not at right-angles to the scanning electron beam and the resulting pictures are, therefore, foreshortened in one direction. The magnification sign symbolizes this.

can be seen in Fig. 4 surrounded by areas in which differential erosion has commenced. This last observation suggests that the polishing procedure that we employed had produced a damaged surface layer of some microns in thickness. This conclusion finds support in the work of BERLIN (1959) and SCHMIDT (1961), and is obviously of considerable significance. For example, this damaged layer is of the same order of depth as the layer that we have analysed by the X-ray emission method mentioned above. The thin sections of enamel prepared by current techniques, such as have been used for contact microradiography and polarized light,

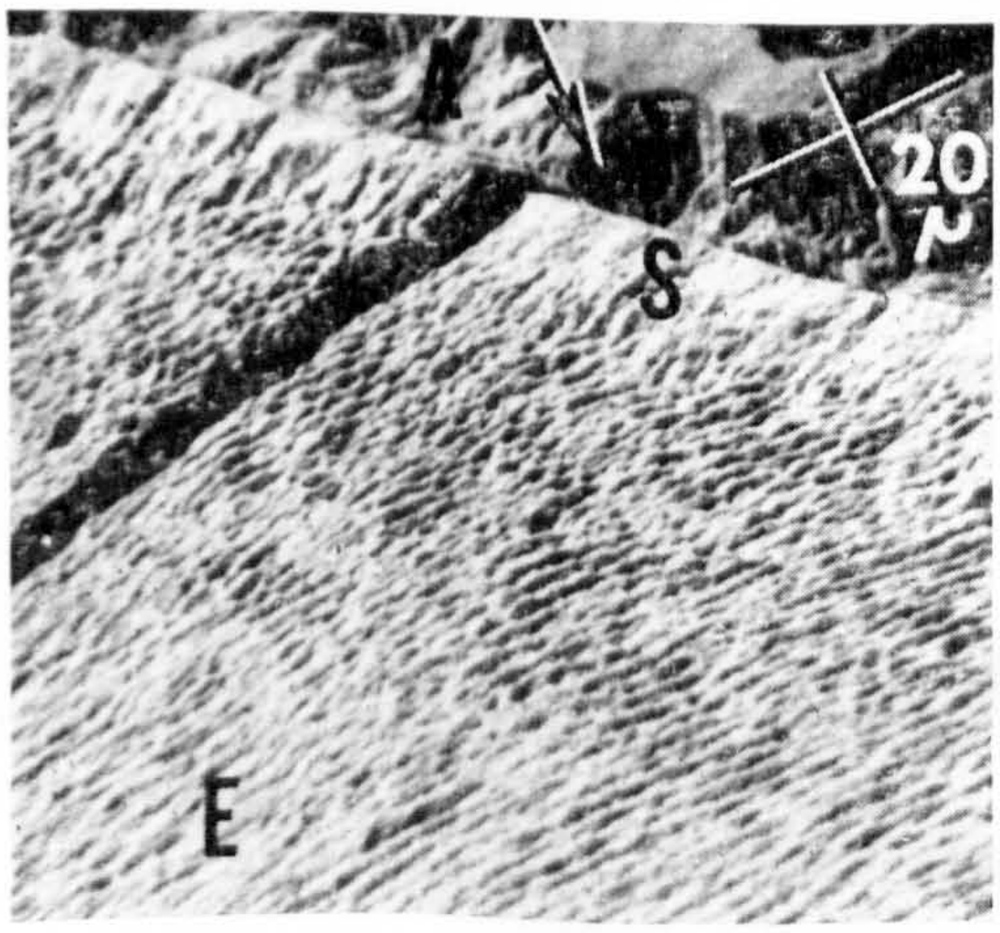

FIG. 5. Scanning electron micrograph of polished argon ion-beam eroded surface of transverse section of *Macropus* cheek tooth showing enamel "surface zone". Enamel below—araldite above. A, araldite; E, enamel; S, true surface zone of enamel.

may have damaged layers amounting to almost the entire thickness of the section.

Accurate measurements in the field of study of elemental micro-analysis and crystallite orientation in relation to histological detail, cannot be expected before the development of a method for the production of thin (1–5μ) parallel sided sections with undamaged surfaces. It is possible that the use of ionic erosion at low angles of incidence may produce a satisfactory surface for this purpose, and we are investigating this possibility.

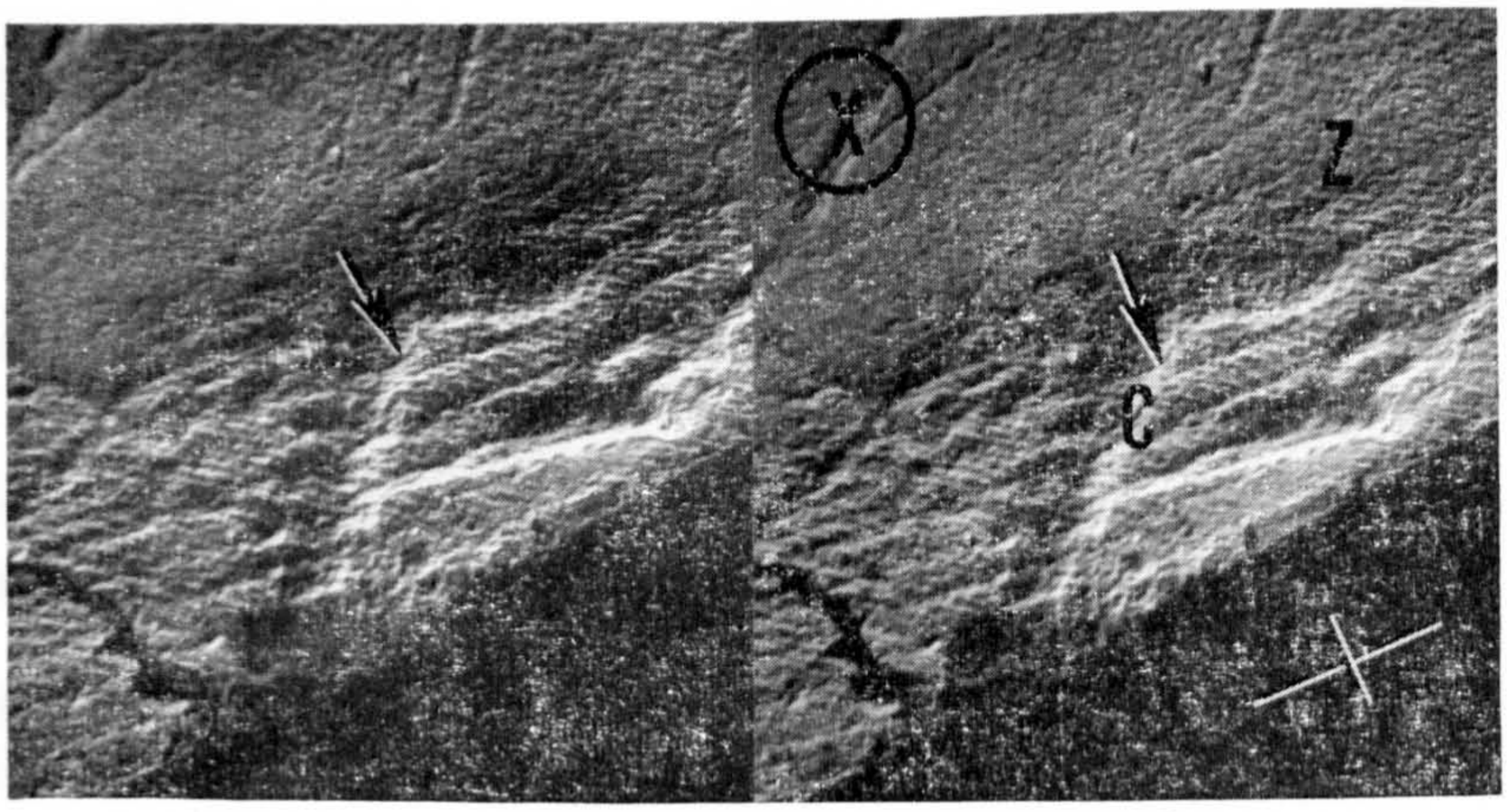

FIG. 6. Scanning electron micrograph stereo-pair of polished argon ion-beam eroded surface of longitudinal section of human premolar through an early interproximal carious lesion. The lesion is surrounded by a zone in which only limited differential etching has occurred. C, centre of carious lesion; X, area in which differential erosion has not yet commenced; Z, zone surrounding carious lesion in which differential erosion was limited.

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THIRD INTERNATIONAL MEETING
IN
FORENSIC
IMMUNOLOGY, MEDICINE, PATHOLOGY & TOXICOLOGY
(LONDON-APRIL 16-24th, 1963)

PLENARY SESSION

IIA

(7)	Estimation of age at death of young human skeletal remains from incremental lines in the dental enamel.	Alan Boyde (London G.B.)

ESTIMATION OF AGE AT DEATH OF YOUNG HUMAN SKELETAL REMAINS FROM INCREMENTAL LINES IN THE DENTAL ENAMEL

A. Boyde

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INTRODUCTION

In determining the age at death of young human skeletal remains, methods which use the state of development of the dentition are more reliable than those using skeletal data, because of the greater constancy of the rate of growth of the teeth. Usually the state of development of the teeth is matched against data compiled from studies of large numbers of normal children: for example, the chart showing development of the human dentition at various ages published by Massler and Schour. The purpose of this paper is to draw attention to another possible method of determining age from the teeth. This method is potentially more reliable because all the information used can be obtained from the remains of the one individual whose age is to be determined. It is, as far as the author is aware, the only "self-contained" method of age determination, in the sense that there is no need to refer to the chronology of development in other individuals. There is, therefore, no need to allow for errors due to deviations from the "normal", which has to be done with methods which depend on making a comparison with data collected from a large number of normal individuals.

If ground sections of teeth are examined with the light microscope, the enamel can be seen to consist of closely packed, narrow (4-8 μ diameter) columnar units, called prisms or rods, (see, Fig. 1) which extend from the underlying, supporting dentine to the surface of the enamel. Enamel is deposited in successive layers from the surface of the dentine outwards, at a rate of 2-8 microns per day in the human. (Schour and Poncher, (1937), Schour and Hoffman, (1938). Massler and Schour, (1941) and (1946)). The daily increments of enamel deposition, (Asper (1916) cited by Massler and Schour (1946)) can be recognised as the "cross striations" of the enamel prisms (Fig. 1). (Retzius (1837)) The

structural basis of these cross-striations is presumably the result of a 24 hour (Circadian Halberg, Halberg, Barnum and Bittner(1959)) physiological rhythm of cellular activity. Certain systemic factors may affect the enamel forming cellular activity and result in the formation of an incremental line or layer which is easily distinguished from the normal(Schour and Massler, 1940). These incremental lines, (Retzius (1837), Berten (1895), Pickerill, (1913)) are commonly called the Striae of Retzius (Figs. 1, 2, 3,) The pattern of these incremental lines is the same in those portions of the different teeth which develop at the same time, and can be followed through the enamel formed in the first few years of life because the periods of growth of the different teeth overlap, one tooth being half formed when the next is just starting. A particularly distinctive incremental line corresponds to the surface of the enamel that obtains at birth. (Rushton, 1933, Schour, 1936). Obviously, this line can only be distinguished in those teeth which are forming at birth, i.e. the deciduous teeth and the first permanent molars. This neo-natal line constitutes a biological landmark which can be used to determine the amount of enamel laid down before and after birth.

Two examples will be quoted of the use of the above information to determine the age at death of child remains. (Figs. 2, 3.)

MATERIALS

Longitudinal ground sections of the first permanent molars and any other permanent teeth present are prepared. The appearance of the cross striations of the enamel prisms may be enhanced by a brief immersion of the section in a dilute acid (e.g. 1% Chromic Acid for 1 minute). The sections should be dehydrated in absolute alcohol, allowed to dry in air, and mounted in thick balsam. These ground sections are examined directly by light microscopy, and also by means of photomicrographs taken at various magnifications and printed at various densities.

METHOD

The total number of cross-striations of the enamel prisms formed from birth (neo-natal line) until death (end of enamel production) is used to provide an estimate of age in days.

Practically, the cross-striations counting must be started along the axis of a small group of prisms which cross the neo-natal line in a first permanent molar. The counting is stopped temporarily when these prisms cross a prominent Stria of Retzius near the surface of the enamel. This Stria is traced cervically to a point close to the enamel-dentine junction and the counting is re-started alone the axis of another prism. Again, when a convenient prominent Stria is reached, the counting is stopped and started again along another prism crossing this Stria further cervically. Naturally, each Stria demarcates enamel formed at the same time, and as has been mentioned above, the pattern can be matched in different teeth. Hence, this counting process can be continued from the first permanent molar to, for example, a permanent incisor, and so on until the last increment of enamel is reached.

The counting is best carried out working directly from the ground sections, using the photomicrographs for convenience in checking off the counts. Thus, each Stria on the micrograph can be assigned an age in days after checking the number of cross striations from a previous Stria along several different prisms, and in different teeth. On most occasions it suffices to measure the average cross-striation interval at each level using an eyepiece micrometer. The distance between Striae is measured is the same way and one thus arrives at an estimate of the number of cross-striations between the two Striae of Retzius with less risk of losing one's position. Counting can be carried out with an accuracy of better than $\pm$ 10% using this method.

The photomicrographs are also employed to match the pattern of the Striae in different teeth, so that the cross-striation counting can be continued from one tooth to the next. The matching is carried out by laying strips cut from a photomicrograph of one tooth over the surface of the other, and vice versa.

DISCUSSION

The validity of the method depends on whether the cross-striations of the enamel prisms are truly daily increments (Massler and Schour, 1946). Some confirmation of this is found in the fact that age estimates arrived at by this means agree closely with those which take account of the state of development of the dentition as a whole. Halberg, Halberg, Barnum and Bittner, (1959)

have shown how many bodily cycles undergo a marked 24-hour periodicity, so that it is not altogether surprising to find evidence of such a cycle "fossilised" in the enamel. The structural basis of the cross-striations has been widely held to a variation of the perfection of mineralisation along the axis of the enamel prisms. Helmcke, Schulz and Scott (1961), in an electron microscope replica study, "noted gentle constrictions along the prisms at the same regular intervals as those assumed by the striations. In addition, certain characteristic arrangements of the ultrastructure components were seen within the prisms. In the central prism core the crystals and organic elements were aligned fairly parallel to the prism axis, while in the cervical region they tended to incline outward, angulation being greatest in the widest portions of the prisms".

Errors in age determination by this method may be caused by the technical difficulties, by a neo-natal arrest of growth of unknown duration and by a post-mortem loss of incompletely mineralised enamel.

Although dental enamel is damaged less than any other body tissue in the processes of post mortem decay, it may be adversely affected, as for example, by a partial demineralisation in an acid environment. In order to be able to handle enamel in such a delicate condition, it is necessary to embed it in a plastic (e.g. polymethylethacrylate) before cutting the ground sections. This embedding process is accompanied by some risk of disruption of the specimen. The cross-striations are often very difficult or impossible to see along the axis of certain groups of prisms. On these occasions it is necessary to revert to using an average cross-striation interval measured on prisms at an adjacent level in the same, or another tooth (see method). Often a number of (~4) cross-striations are involved in one Stria of Retzius. When counting is re-started from the same Stria along a different prism at a more cervical position, it is then possible to introduce an error of two or three cross-striations by continuing the counting from a different level within the same Stria. An internal check on the accuracy of one's counting is provided by finding the same number of cross-striations between corresponding Striae along the axis of any prism in any one of the teeth which developed at the same time.

Schour and Massler (1937) suggest that (at least in dentine) an arrest of growth of 14 days duration occurs after birth. They make no mention of having made an allowance for such an arrest in enamel growth in their later papers (Massler and Schour, 1941, 1946), in which they estimated the appositional life span of the enamel forming cells by dividing the length of the enamel prisms by the characteristic daily rate of apposition at that level. The question as to whether an allowance should be made for a neo-natal arrest of growth in enamel is therefore undecided at the moment.

Enamel is first deposited with approximately 30% of its final mineral content, and in the human a layer of this soft enamel of approximately 50 microns in thickness persists on the surface during its formation. This layer of unknown thickness may be lost before or during the preparation of ground sections, and this will result in the age estimation being too low by an unknawn amount of the order of 14 days.

CONCLUSIONS

The method presented is useful where the most accurate age determination is required, as for example, in those cases where there is a question whether some remains are those of a child which died at birth, or whether it had led a separate existence. It is certainly a useful adjunct to the other available methods. The technical procedures involved are very time-consuming, so that it is unlikely to find a widespread application.

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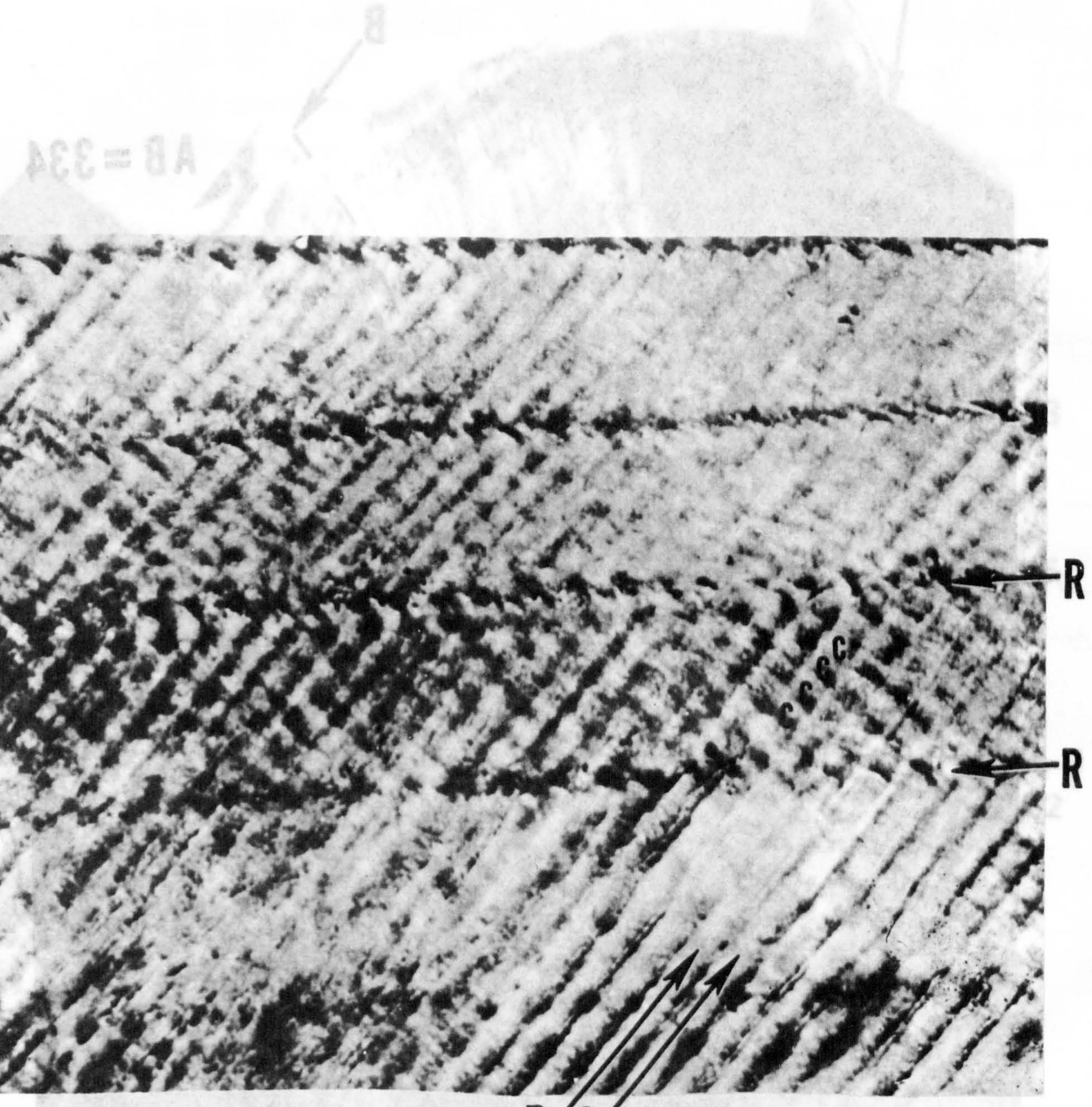
FIGURES

Fig. 1. High power photomicrograph of longitudinal section of enamel Arrows labelled R point to, and are parallel with the Striae of Retzius: arrows P are parallel with the prisms. The cross-striations of the prisms are clearly marked. (c, c, c, c,)

Fig. 2. Longitudinal section through buccal cusp of lower permanent molar (⌜6) of 1 year old child. Enamel formation over this cusp is nearly complete. The total number of cross-striations from the neo-natal line to the surface along group of prisms outlined by the dotted line AB was 334.

Fig. 3. (a) Longitudinal section through mesio-buccal cusp of lower first permanent molar (⌜6) of child aged about $4\frac{1}{2}$ years.
(b) Longitudinal section through labial face of upper central incisor (1̲) of the same child.

The neo-natal line in the ⌜6 is arrowed. Various Striae of Retzius are marked with a number, which is the total number of cross-striations of the enamel prisms counted from the neo-natal line to the Stria (see text). Counting has been transferred from the ⌜6 to the 1̲ and continued, after matching the pattern of the Striae in these two teeth. Enamel formation in the 1̲ is, in fact, just complete, but the process could be continued in another tooth.


B
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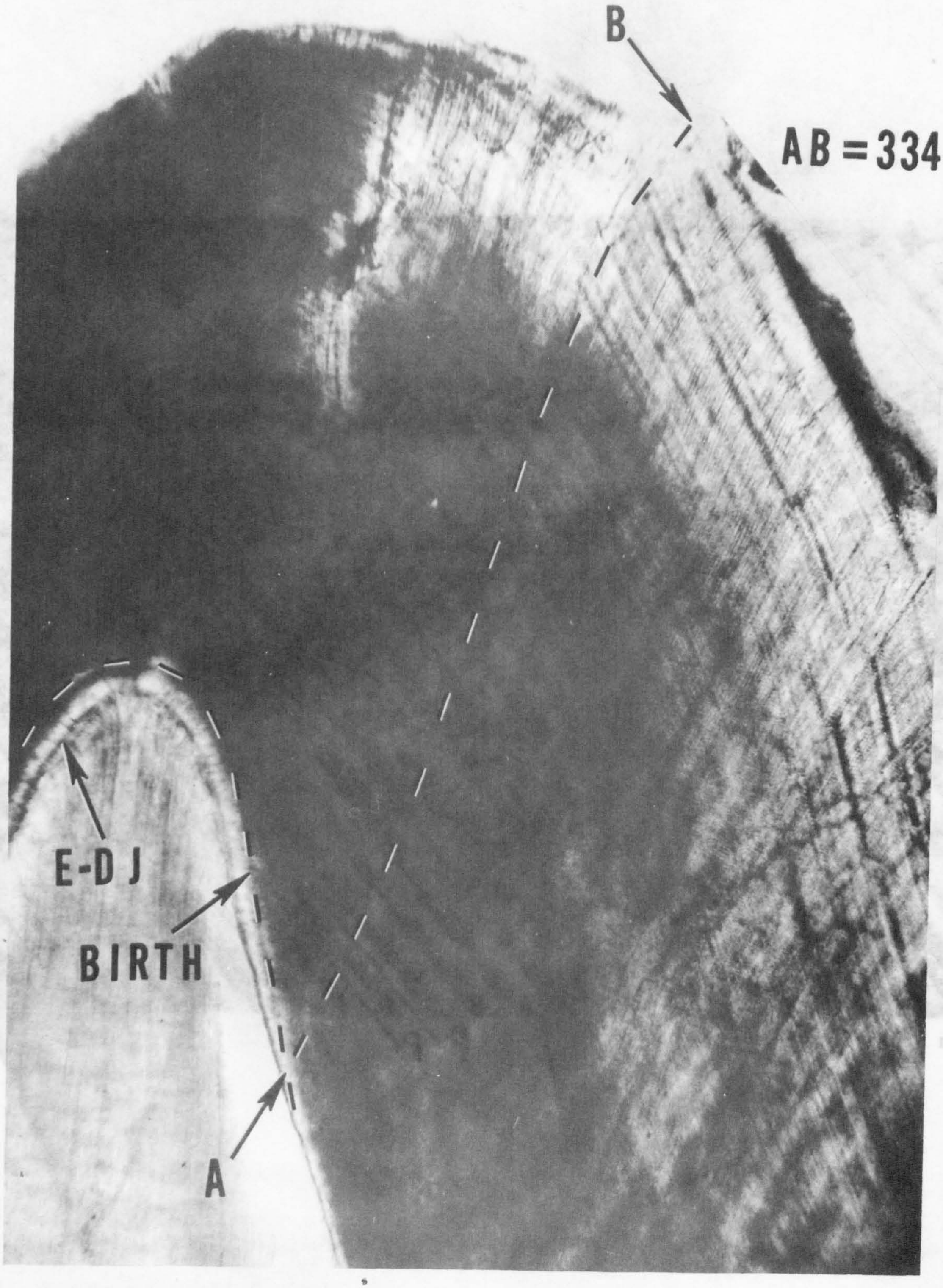
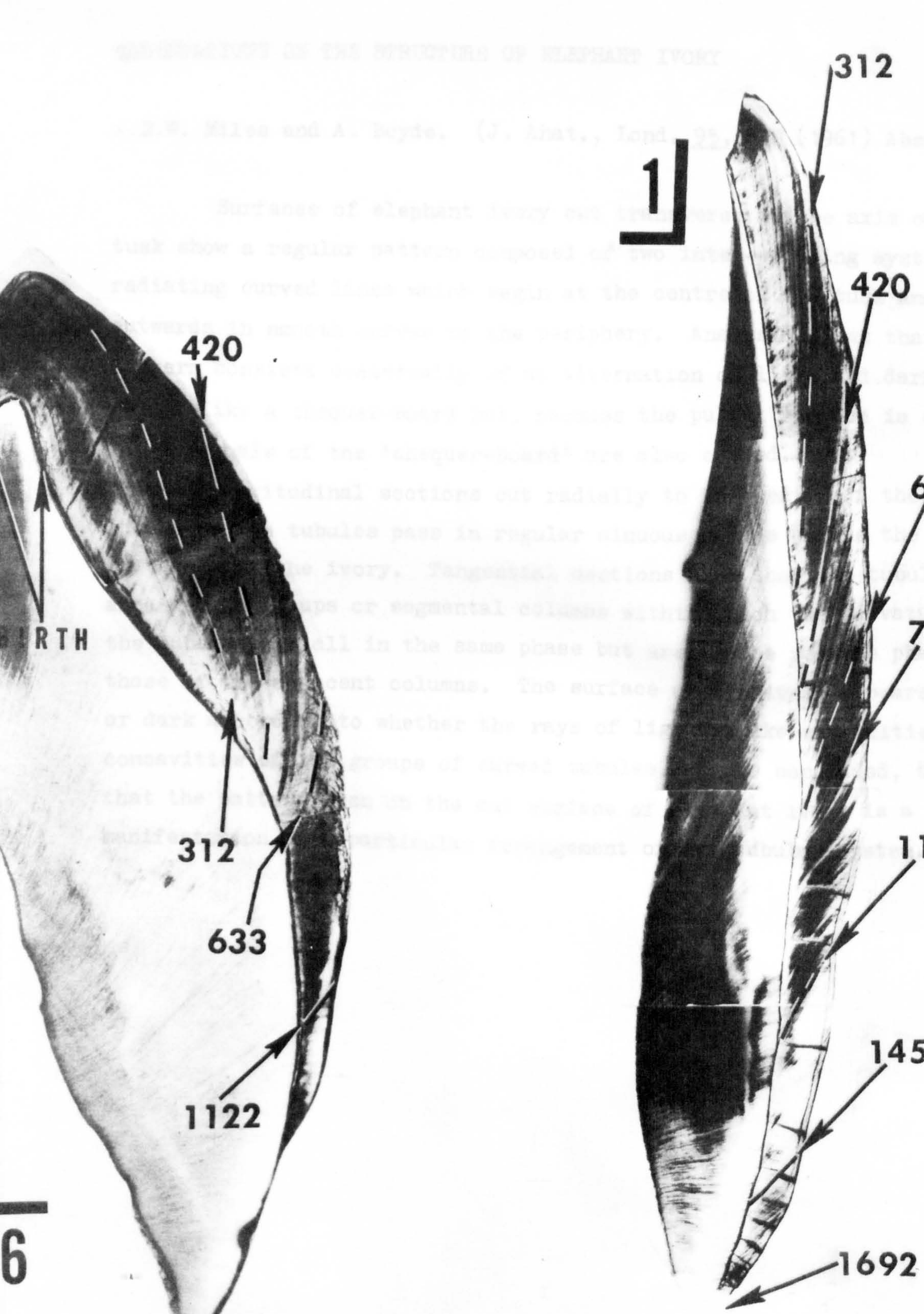


FIG 2



(A) & 3(B)

OBSERVATIONS ON THE STRUCTURE OF ELEPHANT IVORY

A.E.W. Miles and A. Boyde. (J. Anat., Lond. 95, 449 (1961) Abstract)

Surfaces of elephant ivory cut transverse to the axis of the tusk show a regular pattern composed of two inter-crossing systems of radiating curved lines which begin at the centre of the tusk and sweep outwards in smooth curves to the periphery. Analysis shows that the pattern consists essentially of an alternation of light and dark areas rather like a chequer-board but, because the pulpal surface is curved, the diagonals of the 'chequer-board' are also curved.

Longitudinal sections cut radially to the centre of the tusk show that the tubules pass in regular sinuous curves across the thickness of the ivory. Tangential sections show that the tubules are arranged in groups or segmental columns within which the curvatures of the tubules are all in the same phase but are in the reverse phase from those of the adjacent columns. The surface of the ivory appears light or dark according to whether the rays of light strike convexities or concavities of the groups of curved tubules. It is concluded, therefore, that the pattern seen on the cut surface of elephant ivory is a manifestation of a particular arrangement of its tubular system.

Hard-Tissue Sectioning, Surface Finishing, and Powdered Sample Preparation by "Airbrasive" Technique. A.Boyde (1963) J.dent.Res., 42, 1115.

A new surface-finishing technique is required to make further use of physical methods in which only the surface layers of the specimen are studied, e.g. electron-probe x-ray microanalysis. Local high pressures exerted during conventional polishing procedures may result in plastic deformation and/or readherence of abraded materials and cause the formation of damaged surface layers on tooth sections. Machining the surface with very fine abrasive particles in a high-velocity gas stream at a glancing angle of incidence should prevent the "re-packing of abraded material and should not be accompanied by the compression necessary for plastic flow to occur. Only transverse sections were prepared. The tooth was mounted directly onto the spindle of a small electric motor, after drilling a suitable hole along its central axis. The sections were removed quite rapidly by rotating the tooth in a flat air-abrasive stream directed from the closely positioned nozzle of an air-abrasive unit*. This arrangement ensured that the last dust particles to abrade the section surface (i.e., those which finished it) made a glancing angle of incidence with it. The finest abrasive used was 1μ alumina. Sections prepared with 10μ alumina abrasive were suitable - for mounting without further treatment - for light microscopy. Sections of tapering thinness were obtained. Fine, dry, unheated, undistorted powder samples for analysis were collected in a work chamber.

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X-ray Emission Microanalysis of Calcium in Developing Enamel

H. Rosser, A. Boyde and A.D.G. Stewart - J.dent.Res., 43, in press

A scanning electron-probe x-ray microanalyser has been used to measure the CaK_{α} emission from polished longitudinal sections of developing tooth crowns (2 human third molars and 2 rat molars) and apatite fragments embedded in methacrylate. The CaK_{α} emission was recorded from 20μ wide strips perpendicular to, and crossing, the edge of the developing enamel. The level recorded from the apatite standard, under exactly similar conditions, was called 100% CaK_{α} or 100% apatite. The calcium concentration in developing enamel was found to increase linearly with distance from the surface, at an average rate of 2.7% per micron in the human molar cervical enamel and 1.7% per micron in the rat molar occlusal enamel. The maximum concentration (65% apatite) was found at the enamel-dentine junction in the rat. In the much thicker human enamel the CaK_{α} pen traces levelled off at approximately 91% apatite some considerable distance from the enamel-dentine junction. The results do not determine whether the iso-Ca-content contours in mineralising enamel are exactly parallel to the incremental lines, but it is certain that they are not in transverse relation, and there is no second, sudden increase in mineral content. No evidence of a layer of higher mineral content at the surface of enamel during its mineralisation has been found.

Development, Position, and Function of Tubules in Marsupial Enamel

A. Boyde, (1963) J. dent. Res., 42, 1115.

The structure and development of enamel were studied in Macropus, Wallabia rufogrisea, Pseudocheirus convolutor, Acrobates pygmaeus, Trichosurus vulpecula, and Didelphis nudicaudata. Physical continuity between dentin and enamel tubules was confirmed by in vitro dye-diffusion experiments. Trypan blue (1 animal) and tetracycline hydrochloride (4 animals) were injected intraperitoneally in Didelphis. No evidence of these dyes entering even the dentin tubules was found on examining ground sections of teeth extracted after 3 weeks. Methyl blue was inserted into cavities prepared in the pulp of 3 animals' teeth - this dye penetrated as far as two-thirds the length of the dentinal tubules but not into the enamel tubules. In sections of decalcified enamel where some organic matrix was retained, the contents of the tubules were identified as densely basophilic "fibers." Histochemical tests for S-H and S-S groups were negative. Although the tubules are easily seen with the optical microscope, difficulty was experienced in positively identifying them in an electron-microscope study of the developing tissue. (A. Boyde, J. dent. Res., 42, 1081, 1963). They were recognised as such when defects in the close packing of crystallites could be followed through serial sections. The continuity of the enamel and dentin tubules was again confirmed in this way. The enamel tubules formed as a defect during the"filling-in" process in the prism core, and they were not found in the interprismatic regions.